THE ROLE OF IRON IN OXIDATIVE STRESS ACCELERATED ENDOTHELIAL DYSFUNCTION IN CHRONIC KIDNEY DISEASE

T.H.A. HADEIBA

PhD

UNIVERSITY OF BRADFORD

2015

THE ROLE OF IRON IN OXIDATIVE STRESS ACCELERATED ENDOTHELIAL DYSFUNCTION IN CHRONIC KIDNEY DISEASE

Tareg Hadi Ahmed HADEIBA

Submitted for the degree of Doctor of Philosophy

Faculty of Life Sciences

University of Bradford

2015

ABSTRACT

Tareg Hadi Ahmed Hadeiba

The role of iron in oxidative stress accelerated endothelial dysfunction in chronic kidney disease

Key words: Endothelial cell, intravenous iron preparations, oxidative stress, chronic kidney disease (CKD), and cardiovascular disease.

Chronic kidney disease (CKD) is growing global public health problem affecting 1 in 10 adults in developed countries and recognised as an important risk factor for cardiovascular disease (CVD) development. CVD is the main cause of death among CKD patients. Endothelial injury and dysfunction are critical steps in atherosclerosis, a major CVD. Oxidative stress (increased level of reactive oxygen species, ROS) has been associated with CVD development. Intravenous (IV) iron preparations are widely used in the management of CKD mediated anaemia, and have been associated with increased oxidative stress and cellular dysfunction.

This study examined the effect of pharmacologically-relevant concentrations of IV Venofer (iron sucrose) or IV Ferinject (Ferric carboxymaltose, FCM) on primary human umbilical vein endothelial cell (HUVEC) activation/damage and on intracellular ROS generation as well as studying the potential mechanisms responsible. Data from TUNEL assay and Annexin V-FITC/PI staining showed that, IV FCM had no effect, but IV iron sucrose increased HUVEC apoptosis at 24hr. IV iron sucrose inhibited cell proliferation and reduced cell viability. Both compounds induced EC activation through sustained activation of p38 MAPK and up-regulation of ICAM-1 and VCAM-1. Additionally, the compounds induced significant increase in total ROS and superoxide anion production, which was attenuated by the anti-oxidant *N*-acetylcysteine (NAC). P38 MAPK showed up-regulation of pro-apoptotic protein Bax and down-regulation of anti-apoptotic Bcl-2 protein in HUVEC treated with IV iron sucrose and p38 inhibition reversed these effects.

In summary, these results suggest that IV iron sucrose causes more severe EC injury than IV FCM. However, both IV iron preparations induced intracellular ROS and superoxide anion generation in HUVEC leading to EC activation/dysfunction, providing a potential explanation for vascular damage in CKD patients.

ACKNOWLEDGEMENTS

My first thank is to Allah the almighty for giving me the health and knowledge to see the completion of my thesis.

I would also like to give a special thanks to my supervisor, Dr. Anne Graham for all the effort, personal attention, suggestions and endless encouragement and full support she has provided me throughout my PhD study and without whom I wouldn't have been able to produce this thesis. She has been my mentor and has always motivated and encouraged me. Her guidance improved my intelligibility and incisiveness.

I am forever indebted to my parents for their prayers, endless patience and encouragement. In addition, I wish to thank my wife Nahla and my daughter Ruba who have always been very helpful and given me strong hopes at times when I have struggled and for providing a loving environment for me. The two noble ones stood by me all through with patience and tolerance.

I would like to thank all the staff of biomedical sciences department for all their help throughout the research project.

I can not end without thanking my brothers and sisters and all my friends specially Asem for all the support they shown to me.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY OF TERMS

Ab	Absorbance
ACR	Albumin creatinine ration
ADMA	Asymmetric dimethylarginine
ADP	Adenosine triphosphate
AGE	Advanced glycation end product
AMV	Avian Myeloblastosis Virus
AOPPs	Advanced oxidation protein products
AP-1	Activating protein-1
Apaf-1	Apoptotic protease activating factor-1
APS	Ammonium persulfate
ASK1	Apoptosis signal kinase 1
ATP	Adenosine triphosphate
BAEC	Bovine aortic endothelial cell
BH3	Trihydrobiopterin radical
BH4	Tetrahydrobiopterin
BLMVEC	Bovine lung microvascular endothelial cell
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CAD	Coronary artery disease
CAM	Cell adhesion molecule
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic 3,5- guanine monophosphate
CHD	Coronary heart disease

CKD	Chronic kidney disease
CO_2	Carbon dioxide
CRF	Chronic renal failure
CRP	C-reactive protein
CV	Cardiovascular
CVD	Cardiovascular disease
Cys	Cysteine
DAB	3, 3-diaminobenzine
dATP	Deoxyadenosine triphosphate
DC	Diene conjugates
DCF	2', 7'-dichlorofluorescin.
DCFH-DA	2', 7'-Dichlorofluorescin diactate
DDAH	Dimethylarginine dimethylaminohydrolase
DED	Death effector domain
DISC	Death inducing signalling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffer Saline
DPBS	Dulbecco's phosphate buffered saline
DTT	Dithiothreitol
EA.hy926	Endothelial cell line
EC	Endothelial cells
ECL	Enhanced chemiluminescence
ECL	Electro chemiluminescence
ECM	Extra cellular matrix

EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
eNOS	Endothelial Nitric oxide synthases
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
ESRD	End stage renal disease
ET	Endothelin
ET-1	Endothelin-1
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide
FADD	Fas associated death domain
FBF	Forearm blood flow
FBS	Foetal bovine serum
FCM	Ferric carboxymaltose
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FMD	Flow mediated dilation
FMN	Flavin mononucleotide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFR	Glomerular filtration rate
GPCRs	G protein-coupled receptors
GPx	Glutathione peroxidise
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HAEC	Human arterial endothelial cell

Hb	Haemoglobin
HD	Haemodialysis
HepG2	Human hepatoma
HO^-	Hydroxyl anion
$HO^\cdot$	Hydroxyl radicals
hr	Hours
HRP	Horseradish peroxidise
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intracellular adhesion molecule-1
ICAM-2	Intercellular adhesion molecule-2
IMS	Intermembrane space
iNOS	Inducible NOS
IV iron	Intravenous iron
JNKs	N-terminal kinases
K/DOQI	Kidney Disease Outcome Quality Initiative
kDa	Kilo Dalton
KDIGO	Kidney disease improving global outcomes
LDL	Low density lipoprotein
LH	Polyunsaturated fatty acids
LHP	Lipid hydroperoxide
LOOH	Lipid hydroperoxide
LVH	Left ventricular hypertrophy
MAPK	Mitogen activated protein kinases
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage-colony stimulating factor

MDA	Malondialdehyde
MNC	mononuclear cell
MnSOD	Manganese dependent superoxide dismutase
MOMP	Mitochondrial outer membrane permeability
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
MTT	Thiazolyl Blue Tetrazolium Bromide
N_2O_3	Dinitrogen trioxide
N_2O_3	Dinitrogen trioxide
NAC	*N*-acetyl cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium Chloride
NF-κB	Nuclear factor- kappa B
NHS	National Health Services
NICE	national institute for health and care excellence
NKF	American National Kidney Foundation
nNOS	Neuronal NOS
NO	Nitric oxide
NO2	Nitrogen dioxide
NOS	Nitric oxide synthases
NTBI	Non transferrin bound iron
O_2	Oxygen
$O_2^{\cdot -}$	Superoxide anion
O_2^{2-}	Peroxide ion
ONOO-	Peroxynitrite

OS	Oxidative stress
oxLDL	Oxidised low density lipoprotein
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate Buffer Saline
PCR	Protein creatinine ratio
PCR	Polymerase chain reaction
PECAM-1	Platelet endothelial cell adhesion molecule
PMN	Polymorph nuclear Leukocytes
PMVEC	Pulmonary microvascular endothelial cell
PVDF	Polyvinylidene difluoride
PWA	Pulse wave analysis
QRT-PCR	Quantitative real time polymerase chain reaction
RBCs	Red blood cells
RES	Reticuloendothelial system
rhEPO	Recombinant human erythropoietin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RQ	Relative quantification
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sGC	Soluble guanylate cycle

sICAM-1	Soluble intracellular adhesion molecule-1
SMC	Smooth muscle cell
SOD	Superoxide dismutase
sVCAM-1	Soluble vascular adhesion molecule-1
TAE	Tris acetate-EDTA buffer
TBARS	Thiobarbituric acid reactive substances
TBS-T	Tris buffer saline-Tween
TBS-T	Tris buffer saline-Tween
TC	Total cell number
Tdt	Terminal deoxynucleotidyl transferase
TEMED	Tetramethylethylenediamine
TMRE	Tetramethylrhodamine ethyl ester
TNF-R	Tumour necrosis factor-receptor
TNF-α	Tumour necrosis factor-α
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
VCAM-1	Vascular adhesion molecule-1
VLA-4	Very late antigen-4
VSMC	Vascular smooth muscle cells
vWF	Von Willebrand Factor
WHO	World health organisation

CHAPTER 1: INTRODUCTION

1.1. The kidney

1.1.1. Kidney structure

The kidneys are the main part of the renal system, a pair of organs located in a retroperitoneal position on the posterior abdominal wall around the 12th thoracic vertebra. Each kidney about the size of a fist, measuring 10-12 cm in length, 5-7 cm wide, and 2-5 cm thick, and both are identical in structure and function (Snell, 1992).

Each kidney is surrounded by a fibrous capsule and comprises an outer cortex and an inner medulla and contains about one million fundamental structural and functional units called nephrons as shown in Figure 1.1. All nephrons originate in the cortex and consist of glomerular capsule (renal corpuscle) and a tubule system which are related structurally and functionally (Mezzogiorno *et al*., 2002). The glomerulus consists of a network of capillaries situated within Bowman's capsule. The glomerular membrane or glomerular filtration membrane is thought to function primarily as a charge-selectivity filter, allowing proteins to cross on the basis of charge (Steffes *et al*., 2001). The glomerular filtration membrane consists of three layers; a single layer of flattened capillary endothelial cells perforated by fenestrae, a cellular gelatinous layer of basement membrane and an inner epithelial layer of the Bowman's capsule consisting of podocytes which interdigitate to form slits along the capillary wall. These layers of the capillary loop function in parallel, restricting the passage of cells and limiting filtration of macromolecules on the basis of charge, shape, and size (Kitamura and Fine, 1999). Filtered fluid passes into the renal tubule which is divided

into, the proximal tubule within the cortex, the U-shaped loop of Henle extending into the medulla and traversing back into the cortex to form the distal tubule. The latter coalesce to form the collecting ducts which pass through the cortex and medulla and empty into the renal pelvis (Ganong, 2005).

1.1.2. Kidney function

The kidneys are essential organs in the body, controlling the concentration of body fluids. They control water and ionic balance by regulating excretion of water, sodium, potassium, chloride, calcium, magnesium, phosphate, and many other substances, and regulate electrolyte balance of the body (Hsu *et al*., 2011). Also it maintains a stable extracellular environment, which supports the function of all body cells by managing acid-base status. Kidneys produce important hormones such as erythropoietin (EPO), which have a key role in stimulating the bone marrow to produce red blood cells. Furthermore, they produce the enzyme renin, which plays roles in the regulation of blood pressure and kidney function. Another important function is the metabolism of vitamin D to its active form (Parker, 1998; Ferguson and Waikar, 2012).

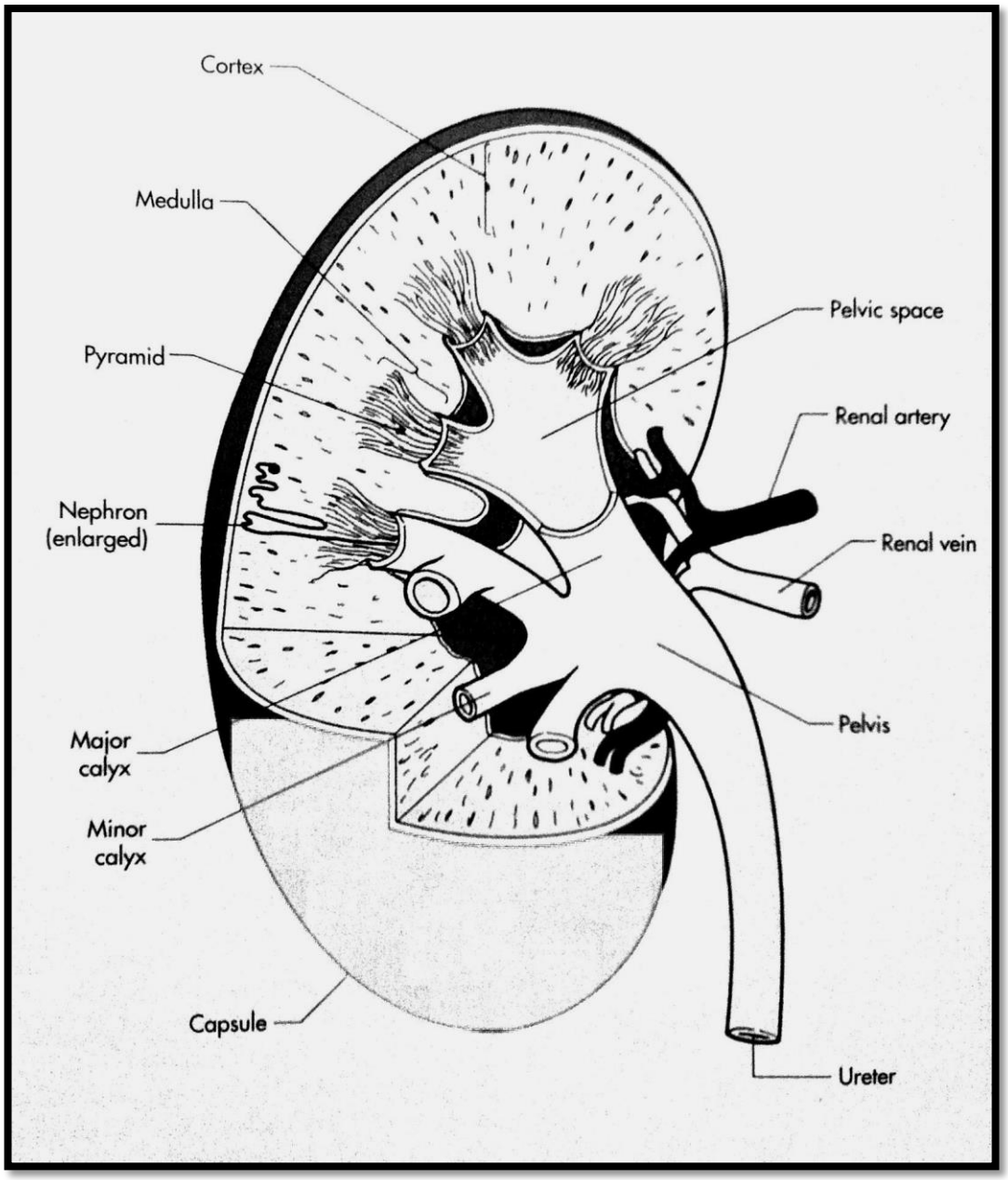


Figure 1.1: Shows the kidney anatomy, taken from Marsh, 1983.

1.1.3. Chronic kidney disease (CKD)

According to the World Health Organisation (WHO, 2005) there were around 58 million deaths worldwide, with 35 million attributed to chronic diseases (World Health Organization, 2005). CKD is increasingly recognized as a major and growing global public health problem which affects about 1 in 10 adults in developed countries (Coresh *et al*., 2007), affecting more than fifty million people around the world with increasing incidence and prevalence, and with both poor outcomes and high cost (Coresh *et al*., 2003; Eknoyan *et al*., 2004). In 2010, it was estimated that CKD cost around £1.45 billion or 1.3% of all the National Health Services (NHS) spending in England (Kerr *et al*., 2012). Approximately, 20 million people or 13% of the general population in America were reported to have CKD (Ardhanari *et al*., 2014) whereas 8.5% of the population in the UK were affected (McLaughlin and Courtney, 2013). In addition, in 2010, the Health Survey for England showed that 6-7% of population over the age of 18 were diagnosed with stage 3 to 5 of CKD. However, there was a clear variation by age and gender as the survey showed that the prevalence of CKD increased to 30% in men and 35% in women aged 75 and over (NICE, 2012). CKD is a complex disease which refers to chronic renal failure or insufficiency, characterized by progressive and irreversible loss of kidney function, usually accompanied by an imbalance of metals, nutrients and electrolytes, eventually leading to total kidney failure also known as end stage renal disease (ESRD) (Oliveira *et al*., 2005). Although, ESRD is a prominent and much feared complication of renal disease, the high mortality rate associated with CKD is mainly due to increased incidence of cardiovascular disease (CVD) (Levey *et al*., 2007),

and is discussed in more detail in section 1.1.5. Blood vessels within the kidney are particularly sensitive to damage so main causes of CKD are atherosclerosis, hypertension and diabetes, which will be discussed in more details in sections 1.1.5.1- 1.1.5.3. However, there are other conditions which can cause CKD such as obesity, inflammatory mechanisms, smoking, infection, family history of kidney disease, age and some medications (McClellan and Flanders, 2003; Ardhanari *et al*., 2014).

1.1.4. CKD stages and classifications

CKD is defined as the presence of kidney damage, and is classified according to the level of kidney function. In 2002, the American National Kidney Foundation (NKF) published the Kidney Disease Outcome Quality Initiative (K/DOQI™) providing guidelines for the evaluation, classification and stratification of CKD (National Kidney Foundation, 2002). Thus, the disease is categorized into five stages of increasing severity, based upon abnormalities of kidney structure or function for more than three months with or without decrease in glomerular filtration rate (GFR), which describe the volume of filtered fluid passing through each kidney functional unit (glomerulus) per unit time with albumin creatinine ratio (ACR) of 30 mg/mmol or higher (Levey *et al*., 2005). However, in order to define an increased risk of adverse results, the UK National Institute for Health and Care Excellence (NICE) advised and updated two important elements of CKD classification that had been published originally by NKF. Firstly stage 3 was divided into two subdivision, stage 3a and 3b as shown in table 1.1. Secondly, NICE also recommended the addition of albuminuria stage after each CKD stage to denote the occurrence of albuminuria, which is defined as ACR of 30

mg/mmol or protein creatinine ratio (PCR) of 50 mg/mmol or higher (NICE, 2014). The subdivision of NICE guideline on classification of CKD has been accepted by the Kidney Disease: Improving Global Outcomes (KDIGO). However, in 2013 KDIGO suggested three categories of ACR for each GFR category as shown in table 1.2 (NICE, 2014).

Table 1.1: The five stages of CKD defined by estimated GFR, taken from NICE, 2014.

Stage	Description	GFR ml/min/1.73m^2
1	kidney damage with normal / increased GFR	Normal/increased GFR≥90
2	Kidney damage with mildly decreased GFR	60 to 89
3a	Mildly to moderately decreased	45 to 59
3b	Moderately to severely decreased GFR	30 to 44
4	Severely decreased GFR	15 to 29
5	Kidney failure	≤ 15

Table 1.2: The three categories of albumin creatinine ratio suggested by KDIGO, taken from NICE, 2014.

ACR category	ACR (mg/mmol)	Terms
A1	<3	Normal to mildly increased
A2	3-30	Moderately increased
A3	>30	Severely increased

1.1.5. Cardiovascular disease in CKD

It is generally accepted that, CVD is a major cause of death globally, affecting both the hearth and blood vessels by atherosclerosis (Perk *et al*., 2012; NICE, 2014). CVD causes 17.3 million deaths annually worldwide and contribute to nearly one third of deaths in England and Wales equating to 191,000 deaths per year (Smith *et al*., 2012). Almost 50% of UK deaths are from coronary heart disease (CHD) also known as coronary artery disease (CAD) and 25% are from stroke; both represent the most clinically significant forms of CVD (Scarborough *et al.,* 2010). Furthermore, CVD has a major cost effects on the UK economy and was estimated to cost the NHS almost £6,940 million in 2003, which increased to £7,900 million in 2010 according to the UK national statistics (NICE, 2014). However, because CVD is frequently associated with CKD, the prevalence of CKD among patients with CVD is even higher than those who do not suffer only from CVD, and many studies have demonstrated that CKD is multiplier for CVD (Go *et al*., 2004). It has been shown that the increased risk of CVD can occur even in early stages of CKD (Di Angelantonio *et al*., 2010). Likewise, CKD is a major, serious and independent risk factor for CVD. It is increasingly apparent that patients with CKD are more likely to die, principally from CVD, than to develop kidney failure or to obtain renal replacement treatment (Keith *et al*., 2004; Rahman *et al*., 2006).

According to the NKF Task Force recommendation, individuals with CKD were to be considered in the “highest risk group” for cardiovascular events, and be treated accordingly for their high risk status (Levey *et al*., 1998). The

NKF Task Force on CVD in CKD issued a report in 1998 showed that, there was high prevalence of CVD in CKD and death from CVD was 10 to 30 times higher in patients with CKD, especially in dialysis patients than in the general population (Foley *et al*., 1998; Foley *et al*., 2005). In addition, patients with combined CVD and CKD have approximately 44% higher mortality rate than patients suffering from CVD (Tonelli *et al*., 2006; Hemmelgarn *et al*., 2009) Furthermore, in patients undergoing dialysis, CVD was shown to be the major cause of death affecting 40% of patients on haemodialysis (HD) (Coronado *et al*., 1998; Al-Dadah *et al.,* 2012). Both the morbidity and mortality of CVD are associated with decline in kidney function, as estimated glomerular filtration rate (eGFR) falls, the incidence and severity of CVD dramatically increases two to fourfold (Chonchol *et al*., 2008; Nakano *et al*., 2010; Gansevoort *et al*., 2013). In addition, higher incidence of CVD is also associated with increased rate of albuminuria (Schmieder *et al*., 2011). Up to 80% of patients with ESRD are expected to die within three years once diagnosed with CHD (Trespalacios *et al*., 2003; Bagshaw *et al*., 2010). Studies showed that Left ventricular hypertrophy (LVH) considered as very strong predictor of CV mortality, is increasingly prevalent in CKD patients even at early stages of the disease due to the high prevalence of hypertension in these individuals (Zoccali *et al.,* 2002; Coresh *et al*., 2007). LVH showed an association with decreased eGFR <30 mL/min/1.73 m2, and estimated as 75% in patients undergoing dialysis (Park *et al*., 2012; Ardhanari *et al*., 2014).

Considerable efforts worldwide are targeted towards characterising the extent of the risk factors associated with CVD, to reduce its impact (Pearson,

1999). The etiology of the increased risk of CVD in CKD is unknown. However, both disorders have shared CVD risk factors, most notably atherosclerosis, hypertension and diabetes (Bleyer *et al*., 2000) and these may play a role in the increased severity of CVD in CKD. Furthermore, inflammation is common in patients with CKD and showed to play pathophysiological role in the pathogenesis of atherosclerosis via contributing to the development of vascular calcifications and EC dysfunction, which can contribute to CVD progression in CKD (Silverstein, 2009). Thus, in order to allow early intervention it is important to assess CVD risk factors in individuals with kidney disease as early as possible after diagnosis (Larson *et al*., 2004).

1.1.5.1. Atherosclerosis in CKD

Atherosclerosis is a disease of the arterial wall, the prevalent form of CVD, and the primary cause of ischaemic heart disease, namely, angina and myocardial infarction. In 2001, a study of mortality in patients with ESRD showed that 20.4% of these patients died of ischaemic heart disease (Rocco *et al*., 2002). Atherosclerosis has been recognized as a common and serious complication in CKD patients (Koike and Nitta, 2011). Additionally, atherosclerotic plaque formation is considered as predictor of CV events in ESRD, and these plaques are present in up to 30% of patients with CKD (Amann *et al*., 2003; Benedetto *et al*., 2008). Furthermore, these plaques contribute to ventricular hypertrophy and heart failure through the association with events, such as arterial stiffness.

1.1.5.2. Hypertension in CKD

Hypertension is a major risk factor, which contribute to cardiovascular risk associated with CKD (Thomas *et al*., 2008) with incidence increasing as eGFR declines (Coresh *et al*., 2001). The prevalence of hypertension among patients with CKD is approximately 70% higher than in the general population and is found in up to 90% of dialysis patients over the age of 65 (Agarwal *et al*., 2003; Coollins *et al*., 2009; Banach and Rysz, 2010). The prehypertensive state is commonly associated with increased presence of inflammatory markers such as C-reactive protein (CRP) and albumin linked to atherosclerosis. Hypertension can damage kidney blood vessels and favours atherosclerosis progression primarily by accelerating the conversion of fatty streaks to raised lesions (McGill *et al.*, 1998). Eventually, vascular lesions can progress to vessel wall necrosis (fibrinoid necrosis, necrotizing arteriolitis, and hyperplastic arteriolosclerosis), which may extend to the glomerulus (necrotizing glomerulitis) (Kumar *et al*., 2003). Both hypertension and atherosclerosis can be closely linked to each other through their effects on EC function due to altered hemodynamic (Chrysohoou *et al*., 2004), and as a consequence of this, ECs become dysfunctional which leads to recruitment of monocytes into the sub-endothelial space where they convert to macrophages full of lipids leading to consequent chemotaxis and aggregation of inflammatory cells (Chrysohoou *et al*., 2004).

1.1.5.3. Diabetes and CKD

Diabetes is a well-known risk factor for CVD with excessive cardiovascular morbidity and mortality, and is associated with adverse outcomes in all stages of CKD, accounting approximately 30% to 40% of patients with CKD

and up to 45% in ESRD (Lea and Nicholas, 2002; Middleton *et al*., 2006). CVD, diabetes and CKD share many risk factors, such as hypertension and obesity. Diabetes patients tend to have higher levels of blood pressure and abnormal cholesterol level, both considered to increase the risk of atherosclerosis, which will increase mortality and morbidity among these patients. Atherosclerosis develops faster and earlier in diabetic patients and affects the systemic and renal large and microvessels (Stout, 1993). In addition, diabetic nephropathy, resulting from high blood glucose levels can lead to abnormal kidney function by damaging the blood-filtering capillaries (Graham *et al*., 2010).

1.1.6. Anaemia and CKD

Anaemia, as defined by the National KDOQI is a haemoglobin (Hb) level of less than 12g/dl for women and less than 13.5g/dl for men (Macdougall *et al*., 2007). It is a relatively common complication of CKD, affecting more than half of all CKD patients (McClellan *et al*., 2004). The 5 stages classification of CKD described above based on the amount of kidney function (glomerular filtration) remaining is very useful because the onset and severity of anaemia has been well correlated with the decline in glomerular filtration rate (GFR). Anaemia occurs in the early stages of CKD patients, such as stage 1 and worsens in those who are in ESRD. In one study, 26.7% of patients with stage 1 of CKD were shown to have anaemia, while 75% with stage 5 CKD were anaemic (Levin *et al*., 1999). In addition, a recent study showed that the prevalence of anaemia increases with progressive stages of CKD, Mehdi *and* Toto found nearly 8.4% of patients with CKD at stage 1 had anaemia compared with the general population figure of 6.3%, while the percentage of

anaemia was 50.3% and 53.4% in patients with CKD at stages 4 and 5 respectively (Stauffer and Fan, 2014). Furthermore, anaemia in patients with CKD and diabetes was 10 fold higher and more severe than in patients with other causes of CKD (Mehdi and Toto, 2009). It is important to identify anaemia in CKD patients because relatively safe and effective therapies are available to correct anaemia and its complications. There are several factors which are likely to contribute to development of anaemia in CKD, such as iron deficiency, decreased production of red blood cells, blood loss and inflammation (Thomas *et al*., 2008; Fishbane *et al*., 2009). However, the primary and important cause is believed to be EPO deficiency (McFarlane *et al*., 2008; Babitt, and Lin, 2012). EPO is a glycoprotein hormone, produced mainly by peritubular capillary lining cells of the kidney cortex (Thomas *et al*., 2007). Under physiological conditions, a basal level of EPO is produced to regulate proliferation, differentiation, and maturation of red blood cells (RBCs) (Thomas *et al*., 2007). Under conditions of hypoxia, kidneys produce EPO to increase RBC production, leading to more oxygen being delivered to tissues (Hodges *et al*., 2007). Anaemia is not only prevalent in patients with CKD but also in patients with diabetes and could significantly contribute to individual's outcomes (Vlagopoulos *et al*., 2005). In addition, low levels of Hb increased risk for development of cardiovascular injury and death in CKD (New *et al*., 2008). Anaemia may potentiate the adverse effects of LVH on cardiovascular outcomes. Anaemia is associated with LVH because LVH increases oxygen demand and anaemia may limit supply (Joss *et al*., 2007). Thus, treating anaemia improves quality of life in patients of CKD, leading to improvements in many areas such as sleep, sexual function, physical

performance and social activities (Lawrence *et al*., 1997; Moreno *et al*., 2000; Dharawhat *et al*., 2009).

1.2. The endothelium

1.2.1. Endothelial cells (ECs)

At one time researchers commonly thought these cells to be simply a non-stick lining of blood vessels, which was believed to function only as anti-blood coagulation and, hence a barrier to diffusion of solutes and nutrients. However, in the last three decades, science has shown that ECs are critical to health; in fact they are now considered to be an active cardiovascular paracrine, autocrine and endocrine organ (Pearson *et al*., 1999; Libby, 2002). The endothelium's location is ideal and considered as an integral part of the vascular system (Davies and Hagen, 1999). ECs are a single layer of cells located at the interface between blood and tissues, separating the vascular wall from the circulating blood and its components, controlling the movement of cells, fluid and small and large solutes such as plasma proteins from the blood to interstitial space and lining of the inner surface of blood vessels as shown in Figure 1.2 (Augustin *et al*., 1994). The layer of ECs constitutes approximately 10^{13} cells, totalling a surface area of 1-7 m^2 and mass of 1 kg in adults (Galley and Webster, 2004). In addition, the endothelium plays an active role in inflammatory reactions, immunity, platelet activity, leukocyte adhesion and regulates vascular homeostasis, through the expression and release of a large number of factors that influence vasomotor tone, fibrinolysis, thrombosis and vascular growth (Vita and Keaney, 2002). Furthermore, the endothelium has emerged as a key regulator of vascular homeostasis particularly in cardiovascular physiology and pathology, in that it

acts as an active single transducer for circulating influences that modify the vessel wall phenotype (Vita and Keaney, 2002). The endothelium is involved in many biochemical pathways in health and disease. EC is able to respond to physical and chemical stimuli, by releasing a wide range of substances such as Nitric oxide (NO), reactive oxygen species (ROS) and angiotensin II and expressing adhesion molecules such as vascular adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and E-selectin (Esper *et al.,* 2006). Some of these secreted substances maintain and regulate vascular flow and vasomotor tone. The ability of endothelium to express antioxidant enzymes such as superoxide dismutase and catalase helps to maintain cellular integrity and function (Leopold and Loscalzo, 2009). The importance of the endothelium in vascular tone was first recognized and confirmed following the discovery that EC produce and release several vasoactive molecules including bradykinin and thrombin that relax or constrict vascular smooth muscle cells, modulating blood flow to any organ (Highsmith, 1998). Vasomotion is modulated by endothelium either by releasing vasodilator substances such as NO and prostacyclin or by an increase in constrictor tone via generation of endothelin (ET) and via conversion of angiotensin I to angiotensin II, a potent vasoconstrictor at the EC surface (Kinlay *et al*., 2001). This vasomotion plays important and direct roles in the balance of tissue oxygen supply and metabolic demand by regulation of vessel tone and diameter, and is also involved in the remodelling of vascular structure and long-term organ perfusion (Schechter and Gladwin, 2003). The endothelium plays a central role in maintaining vascular health by its vital anti-inflammatory and anti-coagulant properties.

Many of these characteristics are mediated by NO, which plays a vital role in EC function (Cines *et al*., 1998).

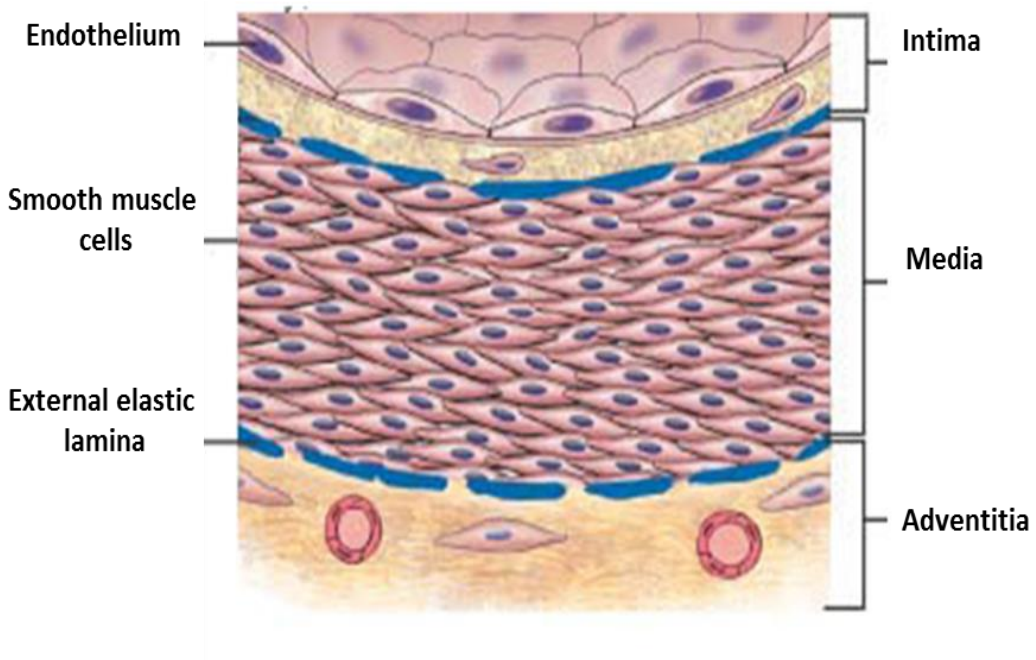


Figure 1.2: Cross-sectional view of endothelium, adapted from Ang and Lumsden, 2001.

1.2.1.1. Endothelial cell adhesion molecules (CAMs)

CAMs are cell surface glycoproteins involved in the binding of cells to each other or to proteins in the extracellular matrix (Cronstine and Weissman, 1993). These molecules are involved in the modulation of leukocyte recruitment and platelet adhesion during thrombosis and inflammation (Springer, 1994). Adhesion molecules are also involved in a number of cellular processes such as, cell growth, differentiation, embryogenesis and cancer metastasis (Bevilacqua *et al*., 1994).

There are a wide range of these molecules expressed by resting and activated ECs, including members of the selectin family both P-selectin and

E-selectin and immunoglobulin (Ig) super family intercellular adhesion molecule-1, intercellular adhesion molecule-2 (ICAM-1, ICAM-2), vascular cell adhesion molecule-1 (VCAM-1) and platelet endothelial cell adhesion molecule (PECAM-1) (Kobayashi *et al*., 2007).

P-selectin is located in granules in ECs and activated platelets. During inflammation, P-selectin can be expressed and moves from the internal location to the surface of the ECs stimulated by a number of inflammatory and thrombogenic mediators such as, histamine, lipid peroxidase and thrombin (Chamoun *et al*., 2000).

E-selectin is a glycoprotein which is not present on un-stimulated ECs. However, is expressed by activated endothelium, synthesized and expressed in response to bacterial toxin and interleukin-1 (IL-1) or tumour necrosis factor-α (TNF-α). E-selectin supports the rolling and initial adhesion of leukocytes, neutrophils, monocytes, eosinophils and some lymphocytes along the endothelium as shown in Figure 1.3 (Xu *et al*., 2000). E-selectin binds to specific ligands located on the surface of the leukocytes such as the tetra saccharide and carbohydrate (Chamoun *et al*., 2000).

ICAM-1 and VCAM-1 belong to the immunoglobulin (Ig) superfamily of proteins (Zhang *et al*., 2010; Cook-Mills *et al*., 2011). The expression of these two molecules is regulated at the level of gene transcription, and is induced by exposure of ECs to stimuli such as IL-1, or bacterial lipopolysaccharide, which are mediated by the transcription factor nuclear factor-kappa B (NF-κB) (Frank and Lisanti, 2008). A low level of ICAM-1 is normally present on healthy resting ECs under basal conditions as well as on lymphocytes and platelets, and *in vitro* study showed that its up-regulation

following stimulation peaks at 12 to 24hr, and persists for 72hr or days as long as the cytokine remains in the culture medium (Rothlein *et al*., 1986; Bevilacqua *et al*., 1994; Frank and Lisanti, 2008). ICAM-1 is also up-regulated in response to oxidative stress such as hydrogen peroxide (H_2O_2), which increases the expression of ICAM-1 mRNA on HUVEC surface (Lo *et al*., 1993). The mechanism was shown to be due to the involvement of H_2O_2 in tyrosine kinase activation, increasing the phosphorylation of Src tyrosine kinase, the initial step in the activation of the MAP kinase pathway upstream of the activation of the transcription factors include activator protein-1 (AP-1) and NF-kB, which results in increased EC permeability (Voelkel and Rounds, 2009). In contrast to ICAM-1, VCAM-1 is expressed only on activated EC, macrophages and myoblasts, stimulated by high levels of oxidative stress and cytokines (Cybulsky *et al*., 1991; Cook-Mills *et al*., 2011). Similar to ICAM-1, VCAM-1 on activated EC appears at 24hr and persists for at least 72hr (Zhang *et al*., 2010). ICAM-1 and VCAM-1 play an important role in the adhesion of leukocytes to EC, which is considered as a crucial step in the initiation of chronic inflammatory diseases such as atherosclerosis (Brooks *et al*., 2002; Van Buul *et al*., 2010). There are a number of steps involved in the adhesion of leukocyte to EC including, capture, rolling, tethering, firm adhesion, and transmigration as shown in Figure 1.3 (Frank and Lisanti, 2008). The main function of ICAM-1 is to facilitate the firm adhesion step while VCAM-1 participates in the slow rolling of leukocytes and transmigration step through binding to very late antigen-4 (VLA-4), a cognate ligand of VCAM-1 expressed on the surface of leukocytes (Frank and Lisanti, 2008). As a result of leukocyte activation both ICAM-1 and VCAM-1 bind to

the up-regulated Leukocyte integrins of the β 1 and β 2 integrin class, this causes the leukocyte to move over the endothelial surface to intercellular junctions where they pass between the ECs and enter the interstitial tissue at the initial stage of inflammation (Van Buul *et al*., 2010).

Up-regulation of both ICAM-1 and VCAM-1 induces intracellular signalling, which results in production of reactive oxygen species (ROS) such as, H_2O_2 and p38 MAPK pathway phosphorylation (Van Buul *et al*., 2010). A study by Deem *et al* showed that, binding of lymphocyte to VCAM-1 across ECs, leads to the activation of endothelial NADPH oxidase for ROS generation (Deem *et al*., 2007). In plasma, both ICAM-1 and VCAM-1 exist as a soluble form (sICAM-1 and sVCAM-1), which can be used as predictive biomarker of disease and correlate with EC dysfunction, atherosclerosis and CHD (Kannel, 2005; Jha *et al*., 2010). Elevated level of sICAM-1 or sVCAM-1 can reflect EC activation and vascular inflammation (Lake and Ridker, 2002), and have been shown to be present in patients with CVD and CKD on HD and with decreased GFR (Stancanelli *et al*., 2010). Furthermore, both sICAM-1 and sVCAM-1 have shown to be independent predictors of mortality in CKD (Suliman *et al*., 2006). sICAM-1 has been shown to be regulated by a number of intracellular signal transduction pathways including the mitogen-activated protein (MAP) kinases (ERK, JNK and p38), and NF-kB (Roebuck and Finnegan, 1999). Elevated sICAM-1 has been found in patients with CKD who have clinical signs of CVD (Colombo *et al*., 2012). In addition, *in vivo* study showed that sICAM-1 and sVCAM-1 were present on EC in atherosclerotic plaques (Mestas and Ley, 2008).

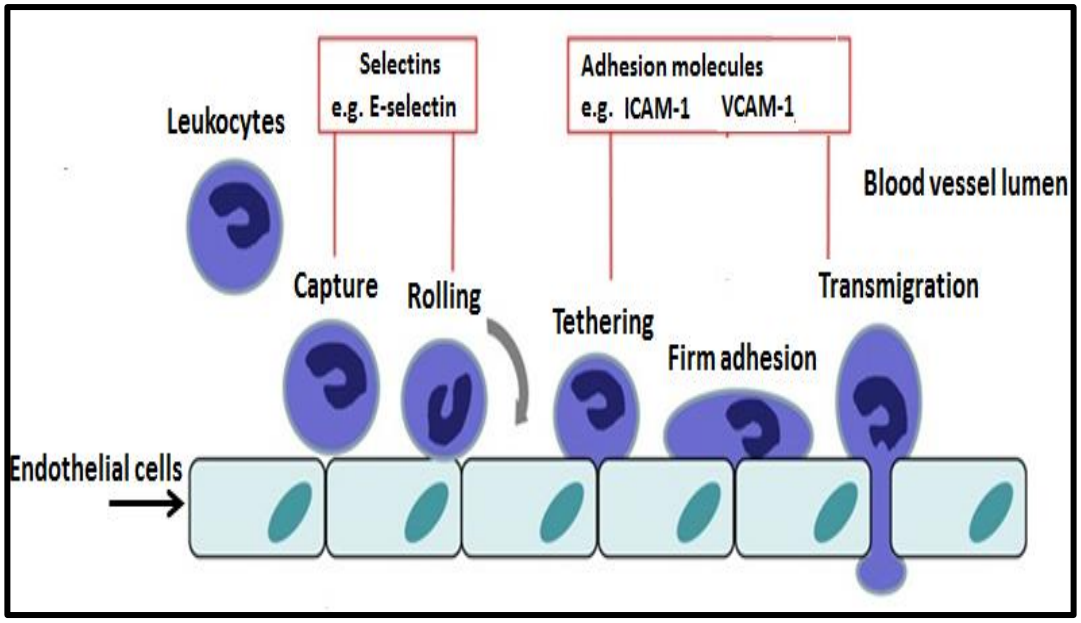


Figure 1.3: Steps of leukocyte-endothelium interaction in inflammatory response: capture (1), rolling (2), tethering (3), firm adhesion (4) and transmigration (5), taken from Warboys *et al*., 2011.

1.2.2. Nitric oxide (NO)

NO officially called nitrogen monoxide is a hydrophilic (Kelm, 1999), and a gaseous lipophilic molecule which as it is uncharged, diffuses across cell membrane (Shimokawa, 1999). NO is a free radical species with an unpaired electron and a very short half-life of 6-10 seconds in physiological media (Burney *et al*., 1999). NO is one of the main vasorelaxing factors and it is considered as an important signalling molecule which can protect the body against high blood pressure, heart failure and thrombosis. In addition, NO has been shown to inhibit adhesion of leukocytes to the vessel wall, which can protect the body against the onset of atherogenesis (Förstermann and Münzel, 2006). NO can be produced by different cell types. However, it is released by ECs mainly in response to shear stress stimuli, triggering

vascular smooth muscle relaxation through activation of soluble guanylate cyclase (sGC), leading to an increase in the intracellular levels of cyclic 3,5-guanine monophosphate (cGMP) and vasorelaxation (Schlossmann *et al*., 2003). NO has also a protective role against atherosclerosis because it inhibits platelet aggregation and adhesion to vascular wall as well as inhibiting vascular smooth muscle cell (VSMC) proliferation through preventing release of platelet-derived growth factors (Napoli *et al.*, 2006).

1.2.2.1. Nitric oxide synthase (NOS)

NO is one produced by a family of Nitric oxide synthases (NOSs) enzymes (Loscalzo and Welch, 1995). NOS exists as three isoforms, endothelial NOS or (eNOS) in ECs, neuronal NOS or (nNOS) in the brain and inducible NOS or (iNOS) in macrophages and other cell types (Alderton *et al*., 2001; Napoli *et al*., 2006), all of which convert the amino acid substrate L-arginine to L-citrulline and NO in the presence of several cofactors, including nicotinamide adenine dinucleotide phosphate (NADPH), Oxygen (O_2), flavin mononucleotide (FMN), haem, flavin adenine dinucleotide (FAD) and tetrahydrobiopterin (BH4) (Ignarro *et al*., 1999). BH4 is an essential cofactor for all NOS isoforms and is involved in the reduction of the haem iron of the enzyme to ultimately form an iron-oxy species that hydroxylates L-arginine to produce NO (Li *et al*., 2011). All NOS enzymes are synthesized as monomers with both reductase domain and oxygenase domain as shown in Figure 1.4A. In the presence of haem, both monomers and reductase domains have the ability to transfer electron from the flavin to the haem of the opposite monomer. NOS couple their haem and start synthesizing NO and L-citrulline when sufficient cofactor BH4 and substrate L-arginine are

present. However, monomers can bind neither the substrate L-arginine nor the cofactor BH4 as shown in Figure 1.4 (Förstermann and Münzel, 2006). Meaning the enzyme is only active as a dimer. eNOS is a tightly regulated enzyme, constitutively expressed in ECs and considered a major weapon of vascular EC to fight vascular diseases (Viaro *et al*., 2000; Ding *et al*., 2007). eNOS can be reduced by cellular metabolism such as increase ROS generation, which the latter will lead to the reduction of NO bioavailability (Ding *et al*., 2007).

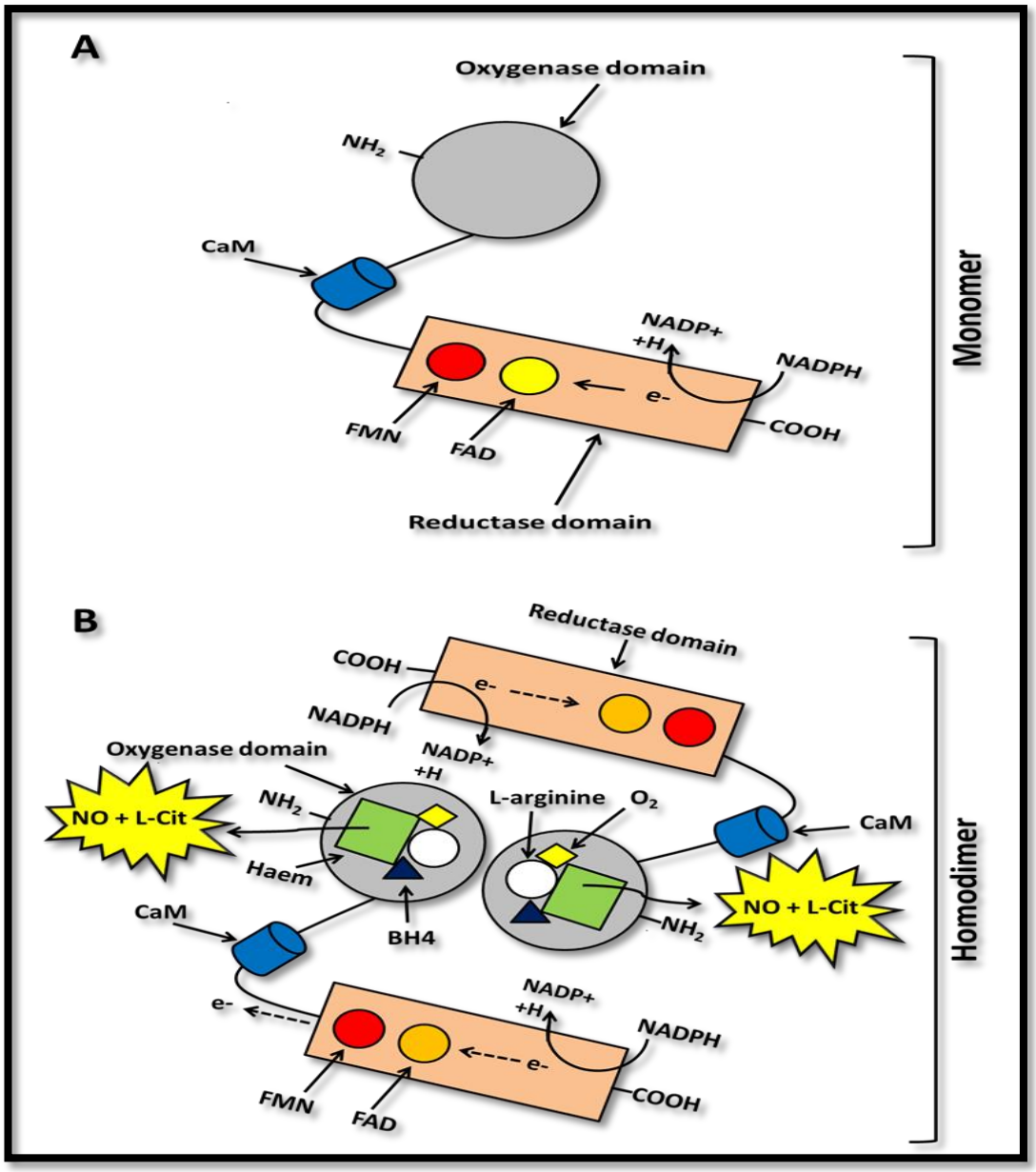


Figure 1.4: The structure of NO production in mammalian cells. **(A):** monomeric structure, each monomer consists of a reductase domain and oxygenase domain. **(B):** dimeric structure, in the presence of haem both monomers and reductase domains have the ability to transfer electron from the flavin to the haem of the opposite monomer. NOS then couple their haem and start synthesizing NO and L-citrulline when sufficient cofactor BH4, O_2 and substrate L-arginine are present,

1.2.2.2. eNOS function and dysfunction (uncoupling)

In the absence of or decreased level of BH4, NOS transfers electrons to molecular oxygen producing superoxide anion ($O_2^{\cdot-}$) and H_2O_2 at the expense of NO, this phenomenon termed eNOS uncoupling (Landmesser *et al*., 2003; Kim *et al*., 2006). Thus, under certain pathophysiological conditions, eNOS can paradoxically become a source of ROS. Generation of superoxide anion by eNOS showed to be involved in a number of experimental and clinical vascular disease states including diabetes, hypertension, and atherosclerosis (Förstermann and Li, 2011). NO can react with superoxide anion to produce the potent oxidant peroxynitrite $ONOO^-$, which is a destructive molecule causing oxidative damage, nitration and S-nitrosylation of nearly all organic molecules such as proteins, lipids and DNA as shown in Figure 1.5 (Förstermann and Li, 2011). $ONOO^-$ in sequence oxidizes the cofactor BH4 to the inactive by-product trihydrobiopterin radical (BH3), and then to BH2 as shown in Figure 1.5 (Najjar *et al*., 2013). The chemical reactivity of peroxynitrite (-O-O-N=O) is due to its easy protonation and the O-O bond cleavage in the trans configuration of its acid form (Ullrich and Bachschmid, 2000). $ONOO^-$ is converted in the presence of excess NO to Nitrogen dioxide (NO_2) and Dinitrogen trioxide (N_2O_3). These species are particularly thiophilic and impact thiol-mediated signalling pathways (Wink and Mitchell, 1998).

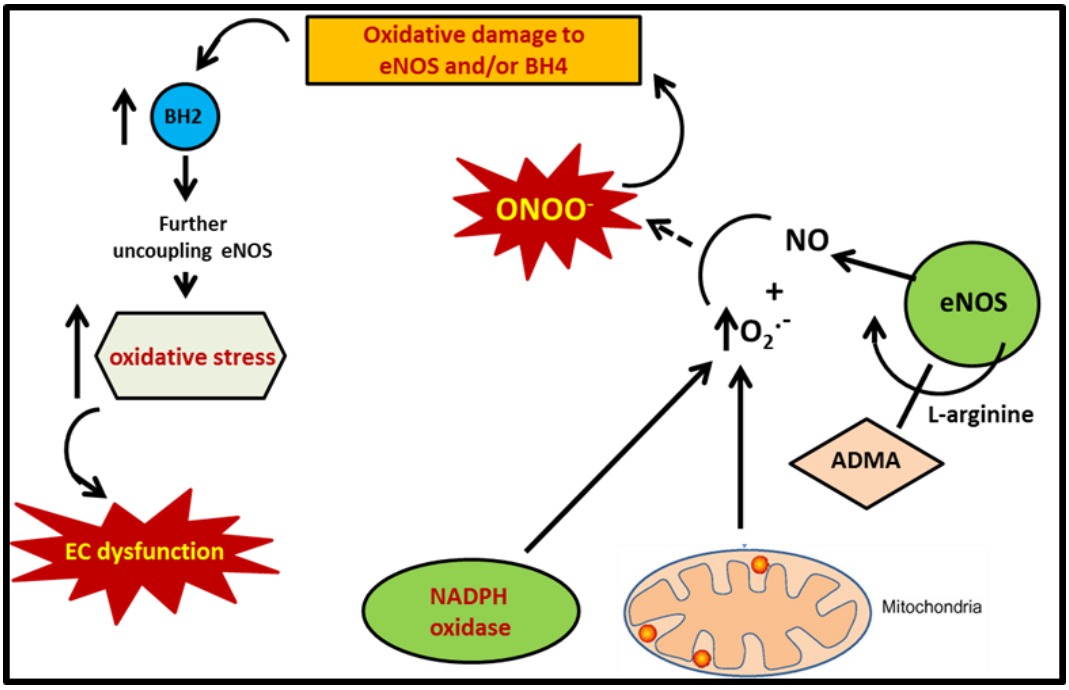


Figure 1.5: The potential mechanisms by which endothelial eNOS uncoupling leads to oxidative stress and EC dysfunction.

1.2.3. Reactive oxygen species (ROS)

Oxygen is a vital component of cellular metabolism (Woodrow, 2007a). However, any defect which results in acute or chronic over consumption of oxygen can lead to the production of free radicals, molecules containing one or more unpaired electrons which have high degree of reactivity, termed ROS. ROS formed by reduction of one or two electrons of the oxygen molecule (Sies, 1986). ROS are oxygen centred molecules which include the radicals such as, superoxide anion, hydroxyl radicals ($HO^{\cdot}$) and NO and non-radicals which act as oxidising agents and/or are easily converted into radicals like, H_2O_2, hydroxyl anion (HO^{-}) and singlet O_2 (Folkes *et al*., 1995). ROS are produced continuously as by-products of many aerobic cellular metabolic pathways that are localized in different cellular compartments such

as mitochondria and peroxisomes (Cerutti, 1985), and are part of the unspecific defence system. In mammalian cells, ROS can be produced by enzymatic reactions including mitochondrial electron transport chain, the cytochrome P450s, xanthine oxidase, NADPH oxidases and uncoupled eNOS (Elahi *et al*., 2009). ROS can be beneficial to living systems as at low concentrations they are necessary for the regulation of several key physiological mechanisms (Valko *et al*., 2006), for instance cell differentiation, regulation of redox-sensitive signal transduction pathways. In immune defence, inflammatory cells such as neutrophils and macrophages have evolved a mechanism to use ROS as a powerful weapon against pathogens (Touyz and Schiffrin, 2004). One further beneficial example of ROS at low concentrations such as superoxide anion or H_2O_2, is the induction of a mitogenic response in variety of cells, resulting in increased rates of DNA replication and cell proliferation (Forkink *et al*., 2010). However, overproduction of ROS clearly can be toxic and damage cellular DNA, lipids and protein (Allen *et al*., 2011). Superoxide anion is considered to be the primary and most important ROS with a unique electronic configuration which has both reducing and oxidizing properties (Miller *et al*., 1990).

Superoxide anion is produced by the addition of one electron to molecular oxygen either through metabolic processes or following oxygen activation by physical irradiation. Superoxide anion causes contraction of smooth muscle cells (SMC); it also rapidly scavenges NO within the vascular wall to reduce its biological half-life (Hattori *et al*., 1991), combining to form $ONOO^-$. While the addition of two electrons yields the peroxide ion (O_2^{2-}) which in biological systems is protonated to give H_2O_2, the latter does not contain an unpaired

electron and therefore is classified as stable ROS (Halliwell *et al*., 2000). In addition, H_2O_2 is not a free radical and does not necessarily interact with tissue through radical reaction directly (Keher, 1993).

Cells contain antioxidant enzymes to keep levels of ROS under control as reviewed by Förstermann (2008), and Nordberg and Arner (2001). These cellular antioxidant enzymes include superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). All of these enzymes can reduce ROS toxicity either by forming a less active radical or by quenching the damaging free radical chain reaction on substrates like proteins, lipids and DNA (Dekkers *et al.,* 1996). SOD represents a group of enzymes that catalyse the dismutation of superoxide anion into O_2 and H_2O_2. Catalase is present in every cell in particular within the peroxisomes. Catalase has the ability to transfer H_2O_2 to water and O_2 and also bind NADPH, which protects the enzyme from inactivation and increases its efficiency. GPx is present in cell cytosol and mitochondria, GPx converts H_2O_2 to water using secondary enzyme such as glutathione reductase (GR) and cofactors such as glutathione (GSH) and NADPH to function as shown in Figure 1.6 (Jones *et al*., 1981).

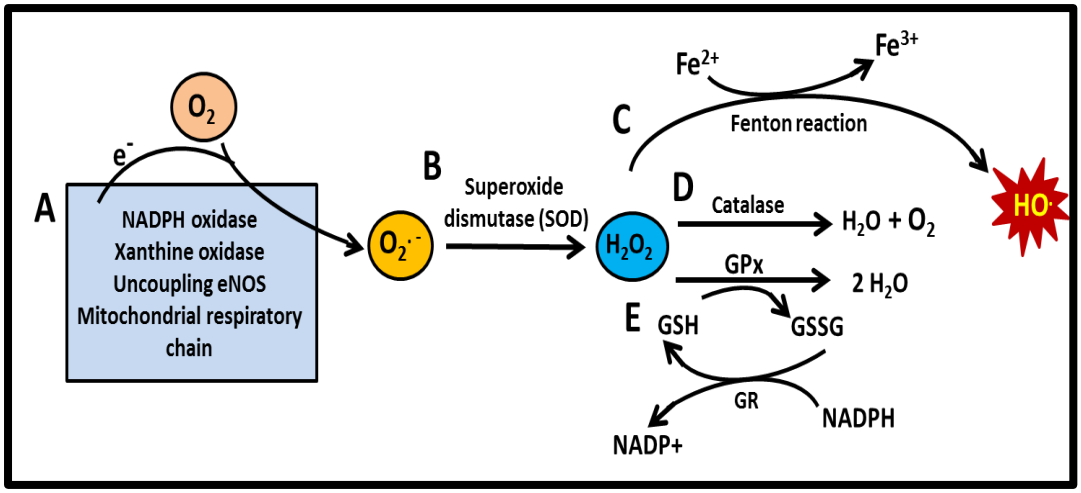


Figure 1.6: The enzyme system involved in the generation of ROS. **(A):** Number of enzymes involved in the production of ROS. **(B):** Overproduction of ROS results in oxidative stress and SOD catalyses dismutation of superoxide into H_2O_2. **(C):** H_2O_2 can be converted to the OH• in the presence of a transition metal, such as iron. **(D):** Catalase has the ability to transfer H_2O_2 to water and O_2. **(E):** H_2O_2 can be detoxified via GPx.

1.2.3.1. Mechanisms of ROS toxicity

When free radicals react with non-radicals, other free radicals can be formed. This enables induction of chain reactions that may be thousands of events long, for instance hydroxyl radicals induces lipid peroxidation of polyunsaturated fatty acids via H_2O_2 abstraction (Kanner *et al*., 1987). Furthermore, the reaction of the hydroxyl radical with aromatic compounds, such as the purine base, guanine in DNA, is processed via H_2O_2 (Mello Filho *et al*., 1984). Thus, ROS can react as both oxidising and reducing agents. Although the initial free radical produces only local effects, secondary radicals and degradation products can have biological effects distant from the site where the first free radical was formed. However, when two free

radicals react with each other, a stable molecule may be formed (Cheesman and Salte, 1993). These explain the eventual termination of free radical-induced chain reactions.

Superoxide anion is potentially toxic. It may directly influence local homeostasis by oxidising catecholamines, or it can be transformed into the hydroxyl radical via the Haber-Weiss reaction (Folkes *et al*., 1995). In contrast, H_2O_2 *per se,* is not especially toxic to cell macromolecules. However, it can cross the membranes of cells, which is potentially essential because the extracellular environment possesses few antioxidant defence mechanisms (Flora, 2009). In the presence of low concentrations of transition metal ions, such as iron and copper, hydroxyl radicals are formed from H_2O_2, via the Fenton reaction as described below. Alternatively, H_2O_2 can interact with superoxide anion to produce the hydroxyl radical, by Haber-Weiss type reaction.

$$\text{Ferric iron } (Fe^{3+})/\text{ Cu (II)} + \bullet O_2^- \rightarrow \text{Ferrous iron } (Fe^{2+}) + O_2$$

$$Fe^{2+}/\text{Cu (I)} + H_2O_2 \text{ ----> } Fe^{3+} + HO^{\cdot} + OH^-$$

Due to the charged nature of the superoxide anion it is more concentrated in the intracellular compartment. As a result, hydroxyl radicals are produced predominantly from H_2O_2 by Haber-Weiss reaction in the intracellular compartment whereas the Fenton reaction is more important in extracellular compartments (Fridovich, 1983).

1.2.3.2. Sources of ROS in the Endothelium

ROS such as H_2O_2 and superoxide anion may be produced within the endothelium similar to other non-phagocytic cells such as SMC and fibroblasts, at relatively low levels in response to extracellular signals or stimuli. The principal sources of ROS in ECs are the mitochondria-specific manganese-dependent superoxide dismutase (MnSOD), NADPH oxidase, uncoupled eNOS (described in section 1.2.3.2), xanthine oxidase and cytochrome P450 (Li and Shah, 2004; Holley *et al*., 2011).

1.2.3.2.1. The mitochondria as a generator of ROS

Oxidative phosphorylation is the most critical function of mitochondria. This system contains four large multi-enzyme complexes, known as complexes, I, II, III, IV. Mitochondrial oxidative chain is a process in which electrons are extracted from NADH and FADH and transferred to molecular O_2 through the chain of the four enzymes which is subsequently used to ensure the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP). ATP production requires molecular O_2 reduction to H_2O_2 (Li and Shah, 2004). These enzymes transfer electrons along the electron transport chain (ETC) which generate a proton gradient, enabling generation of ATP (Turrens, 2003). Under physiological conditions, the mitochondria respiratory chain is the major source of ROS, namely superoxide anion generated as a by-product (Puddu *et al*., 2005). Superoxide anion has been implicated in the pathophysiology of CVD (Kovacic *et al*., 2005).

$$e^- + O_2 \rightarrow O_2^-$$

$$2e^- + O_2 + 2H^+ \rightarrow H_2O_2$$

Since the mitochondria represent the most powerful cellular source of ROS in the body, it appears that mitochondria are primary target for their deleterious effects (Dröge, 2002). Mitochondrial DNA (mtDNA), which encodes for the majority of protein products that are essential components of the mitochondrial respiratory chain, is particularly sensitive to oxidative damage. Exposure of ECs to H_2O_2 or $ONOO^-$ results in preferential damage to mtDNA over nuclear DNA, leading to mitochondrial dysfunction with decreased mitochondrial protein synthesis (Ballinger *et al*., 2002). Oxidative damage to the mitochondria can lead to decreased oxidative energetic capacity via impaired oxidative phosphorylation and enhanced generation of intracellular ROS.

1.2.3.2.2. NADPH oxidase

In the vasculature and kidneys, NADPH oxidases are the primary source of ROS (Babior *et al*., 2002; Feng *et al*., 2010). These are a family of multiple subunit complex enzymes that generate superoxide by the reduction of O_2 using NADPH or NADH as the electron donor, and the superoxide anion can be converted to H_2O_2 by SOD or to highly reactive hydroxyl radical or to peroxynitrite by reacting with NO. NADPH oxidase was originally discovered in phagocytes (neutrophils), where it plays an essential role in non-specific host defence against microbial organisms (Babior *et al*., 2002). Vascular NADPH oxidase consists of membrane integrated cytochrome b_{558} (Griendling *et al*., 2000), and comprises seven isoforms, Nox1, Nox2, Nox3, Nox4, and Nox5, and Duox1 and Duox2 (BelAiba *et al*., 2007). In addition to

this membrane- associated catalytic core, the four cytosolic subunits ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and the small GTP-binding protein Rac1or (Rac2)), are essential for the regulation of oxidase activity (DeLeo *et al*., 1998).

Cytochrome b_{558} is comprised of two subunits, $p22^{phox}$ and the catalytic $gp91^{phox}$ also known as Nox2 (Babior, 2004). In unstimulated cells, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ exist in the cytosol, whereas $p22^{phox}$ and the catalytic $gp91^{phox}$ are in the membrane, as a heterodimeric flavoprotein, cytochrome b_{558} (Paravicini and Touyz, 2008). However, on stimulation, $p47^{phox}$ becomes phosphorylated and cytosolic subunits form a complex that translocates to the membrane, where it associates with cytochrome b_{558} to assemble the active oxidase, which transfers electrons from the substrate to molecular O_2, forming superoxide anion and secondary ROS (Paravicini and Touyz, 2008). Although initially thought to be limited to phagocytes, it is now appreciated that many components of this oxidase including the catalytic Nox2 are expressed in non-phagocytes. For instance, Nox2, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$, and Pac1 are all expressed in ECs (Geiszt, 2006). In the vascular wall, CVD risk factors such as hypertension and diabetes, which are known to contribute to CKD stimulate ROS production, particularly of superoxide through the increase activity of NADPH oxidase, producing $ONOO^-$ as described in section 1.2.3 (Förstermann and Münzel, 2006; González *et al*., 2014). This uncouples eNOS and increases oxidative stress, which can contribute to vascular damage due to reduced bioavailability of NO shown in Figure 1.7 (Kuzkaya *et al*., 2003; Förstermann and Münzel, 2006; Joshi *et al*., 2013).

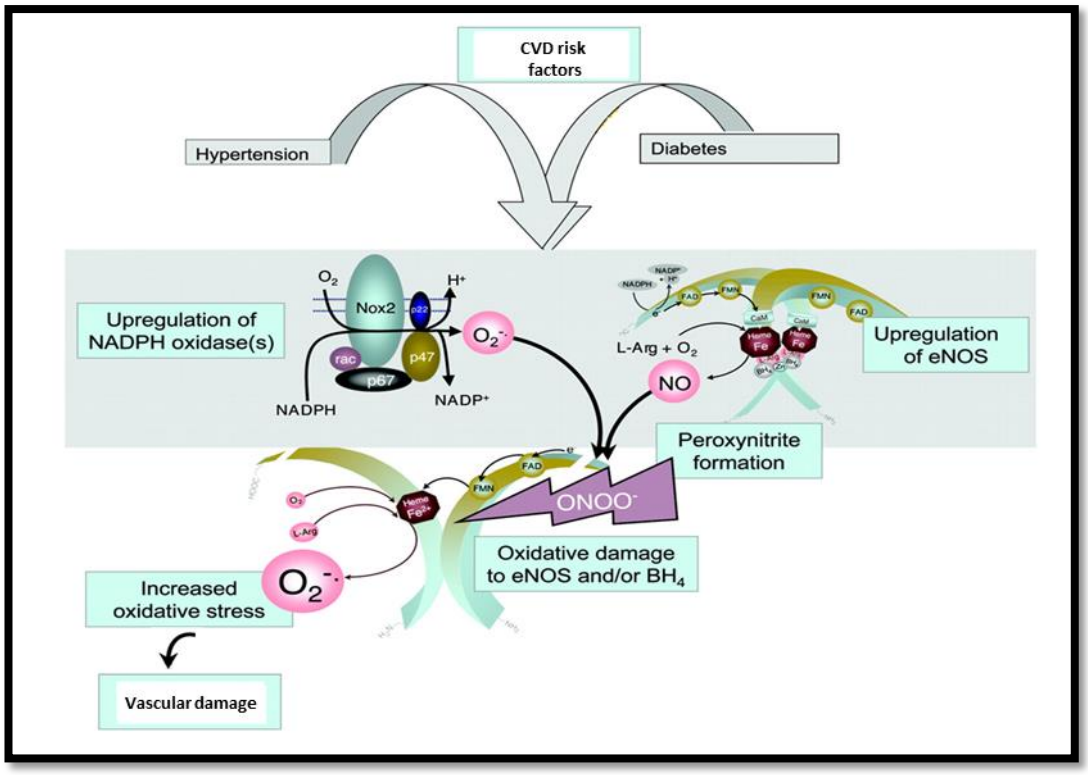


Figure 1.7: The role of CVD risk factors such as, hypertension and diabetes in NADPH-induced ROS generation, adapted from Förstermann and Münzel, 2006.

1.3. Oxidative stress, EC dysfunction and atherosclerosis development

The term oxidative stress defined as high levels of ROS, which can cause cells and tissue damage (Sies, 1997; Dayem *et al*., 2010). High oxidant stress occurs when the formation rate of ROS exceeds the capacity of physiological antioxidant defence mechanisms (Higashi *et al*., 2009). The extent of oxidative stress has been variably determined by measurement of a decrease in total antioxidant capacity, through depletion of individual antioxidants such as vitamin E, vitamin C or GPx (Ogino and Wang, 2007).

Atherosclerosis is a chronic low grade inflammatory disease (Ross, 1995; Dessì *et al*., 2013), and EC dysfunction occurs at an early stage (Totoson *et*

al., 2014). EC dysfunction involves abnormalities in the homeostatic function of vascular endothelium including disturbed vascular tone, haemostasis, and inappropriate inflammation (Cai and Harrison, 2000). It is evident that during CVD process, ROS overcome antioxidant defences, leading to oxidative stress (Davidson and Duchen, 2007). EC dysfunction is initiated or defined by reduced NO bioavailability and increase in endothelium-derived contracting factors like endothelin-1 (ET-1) due to increased oxidative stress, which plays a key role in the reduction of NO biological activity via the up-regulation of eNOS uncoupling as described in section 1.2.3.3 (Tziomalos *et al*., 2010; Cavieres *et al*., 2014). These abnormalities lead to impairment of endothelium-dependent relaxation of blood vessels, eventually resulting in cardiovascular disorders (Messner and Bernhard, 2014). In addition, under inflammatory conditions, such as atherosclerosis, activated leukocytes are attracted to the site of injury and facilitate the production of ROS which worsen the injury (Papatheodorou and Weiss, 2007). Furthermore, oxidative stress has been shown to increase vascular endothelial permeability, and this was supported by *in vitro* study in which direct treatment of bovine pulmonary artery ECs with H_2O_2 increased trans-endothelial permeability. H_2O_2 altered the vascular permeability via increased phosphorylation of tyrosine kinases, which potentiated binding of neutrophils on endothelium (Usatyuk *et al*., 2003; Vogiatzil *et al*., 2009). The early events of atherosclerosis include sublethal changes in EC, where the endothelium shows increased permeability to plasma proteins and lipoproteins, like low density lipoprotein (LDL), albumin and fibrinogen (Phinikaridou *et al*., 2012). LDL can be a target for ROS which is very cytotoxic for EC, and induces

inflammatory cells recruitment to the endothelium, a key event in atherogenesis (Spickett *et al*., 2013). LDL binds to extracellular matrix proteins of the sub-endothelial space and accumulates in the artery wall, where it can be oxidised (oxLDL) (Milioti *et al*., 2008). EC and leukocytes release chemokines that cause proliferation and migration of SMC from the media to the intima (Gosmanova and Le, 2011). SMC help in accumulation of collagen and proteoglycan which are essential factors for the plaque stability (Braunersreuther *et al*., 2007). Two phenotypes of plaque have been identified, those with thick fibrous cap and small lipid core and termed stable plaques which do not pose much danger of rupture inducing atherosclerotic complications. In contrast, unstable plaques are characterized by large lipid core and thin fibrous cap and are prone to rupture, inducing thrombosis leading to acute vascular events (Fuster *et al*., 1999; Mallat and Tedgui, 2006).

Oxidative stress is implicated in atherosclerosis (Hilliwell and Gutterige, 1999; Son *et al*., 2011). Several lines of evidence indicate that oxidative stress induces EC activation and dysfunction by altering different pathways (Cai and Harrison, 2000). In the presence of increased ROS, ECs lose their protective phenotype and express proinflammatory molecules such as ICAM-1, monocyte chemotactic protein-1 (MCP-1) and VCAM-1, which are regulated or controlled by a redox-sensitive transcriptional regulatory protein NF-κB as described in section 1.2.1.1 (Gosmanova and Le, 2011). In ECs, NF-κB is a prime target for ROS, has been shown to be redox sensitive and to be regulated by intracellular redox state (Meyer *et al*., 1994; Joshi *et al*., 2013). In EC, oxidative stress particularly H_2O_2 plays a regulatory part in

other aspects of intracellular signalling, and has been shown to mediate activation of Mitogen activated protein kinase (MAPK) family members like p38 MAPK, which play a role in the activation of NF-κB (Ushio-Fukai *et al*., 1998; Foncea *et al.,* 2000). Growing evidence indicates that exposure of ECs to ROS induces apoptosis, leading to EC loss and results in atherogenesis and a procoagulative state (Dimmeler and Zeiher, 2000). Mechanisms of apoptosis are regulated by ROS through a variety of stimuli in EC including, oxidized LDL, angiotensin II, high glucose, and TNF-α, which are inhibited by *N*-acetyl cysteine (NAC), vitamins C and E, which will be discussed in detail in section 1.5 (Dimmeler and Zeiher, 2000). There is a well-established relationship between both oxidative stress and hypertension as angiotensin II signalling is also an important inducer of superoxide production in the vascular wall through up-regulation and activation of NADPH oxidase (Zalba *et al*., 2001).

1.4. Role of oxidative stress and EC dysfunction in CKD

As described in section 1.1.5, CKD patients are at greater risk of CVD and these patients are more likely to die from CVD than to develop to ESRD (Dhaun and Webb, 2013). Both dysfunction of ECs and oxidative stress are present in patients with moderate to severe CKD, which may explain the increased cardiovascular risk in CKD patients (Martens *et al*., 2011). Vascular cells, activated macrophages and glomerular cells are renal source of ROS, which all can play a role in renal diseases such as glomerulonephritis and tubulointerstitial nephritis; these suggest that the kidney may be particularly susceptible to oxidative stress (Ichikawa *et al*.,

1994). A number of oxidative stress biomarkers such as lipid peroxides, malondialdehyde (MDA) and oxLDL (Agarwal, 2004; Diepeveen *et al*., 2004)**,** and advanced oxidation protein products (AOPPs) were shown to be increased in plasma of CKD patients as a well as in those on dialysis (Stenvinkel *et al*., 2008). A study by Costa-Hong *et al* showed that, oxidative stress markers correlate with EC dysfunction in patients at stage 5 of CKD who have no sign of CVD. The results showed significant increases in thiobarbituric acid-reactive substances (TBARS), a marker of lipid peroxidation and a reduction of brachial-artery flow-mediated dilation, which indicate that increased oxidative stress could adversely affect endothelial function, and eventually cause CVD in CKD patients (Costa-Hong *et al.,* 2009). Furthermore, increased signs of oxidative stress and EC dysfunction are implicated with decreases in renal function, and showed to be present in early stages 1 and 2 of CKD (Tsuchikura *et al*., 2010). A study showed that levels of AOPP was significantly increased in early stages of CKD, and was gradually elevated with renal failure progression, as GFR was inversely associated with plasma concentrations of AOPP (Witko-Sarsat *et al*., 1998; Tsuchikura *et al*., 2010)**,** suggesting that decline in renal function may have direct effect on worsening of endothelial function (Khaira *et al*., 2011). Moreover, perfused mesenteric arteries from rats that underwent renal mass reduction have impaired vasodilation in response to acetylcholine within 3-10 days after surgery. This impairment was restored by treatment with SOD, indicating that at early stages of CKD, oxidative stress plays a role in EC dysfunction (Martens and Edwards, 2011).

One of the major effects of oxidative stress is the reduction in NO biological activity (Costa-Hong *et al*., 2009). Reduced NO bioavailability has been shown to contribute to glomerular hypertension and accelerate renal damage leading to more rapid progression of CKD (Baylis, 2008). Cross *et al* investigated the contribution of oxidative stress to the decreased NO bioactivity detected in ESRD patients, and the authors found there was a decrease in acetylcholine-induced forearm blood flow in these patients, which was improved by infusion of antioxidant vitamin C, demonstrating EC dysfunction was due to increased oxidative stress (Cross *et al.,* 2003). The presence of inflammation is a well-documented factor influencing the development of oxidative stress in dialysis patients through the promotion of hypertension which is known as risk factor of both CVD and CKD, causing EC dysfunction (Samouilidou *et al*., 2003).

Asymmetric dimethylarginine (ADMA) is a naturally occurring amino acid that circulates in blood and is excreted in urine, and is an endogenous inhibitor of all types of NOS (Memon *et al*., 2013). Some of ADMA is cleared by renal excretion from the circulation (Jacobi *et al*., 2014), However, it has been reported that, dimethylarginine dimethylaminohydrolase (DDAH) enzyme which contain two isoforms DDAH 1 and DDAH 2, is responsible for the clearance of the vast majority of ADMA extracting it from the circulation, through metabolizing it to L-citrulline and dimethylamine and therefore DDAH is able to determine NO bioavailability (Janssen *et al*., 2013). Reduced clearance of ADMA in renal failure is associated with EC dysfunction (Lin *et al*., 2002). Elevated ADMA levels have been linked with an increased risk of CVD events and total mortality (Sibal *et al*., 2010). Oxidative stress may

increase synthesis of ADMA by stimulating the expression of methyl transferases or by reducing DDAH activity (Zhang *et al*., 2010). In addition, it was reported that increased levels of plasma ADMA were present in patents with CKD and were associated with higher intima-media thickness and cardiovascular events (Zoccali *et al*., 2001).

Both elevated ADMA and high oxidative stress have been reported to be risk factors for EC dysfunction as a result of reduced activity of NOS (Hackenhaar *et al*., 2014). In human ECs such as HUVEC, oxidative stress has been shown to decrease the activity of DDAH, resulting in an increase in ADMA concentrations. Therefore, an increased production of ROS could be the reason for increased ADMA levels (Sibal *et al*., 2010). In CKD, levels of plasma ADMA may increase from 3 to 9 fold, which leads to inhibition of NO production by 30-70% (Lu *et al*., 2011). The reduction of NO production and impairment of DDAH ability to metabolize ADMA, leading to impaired endothelium-dependent vasodilation and elevated platelet aggregation may explain why ADMA can be used as a cardiovascular marker, and may predict cardiovascular events in ESRD patients as shown in Figure 1.8 (Lu *et al*., 2011).

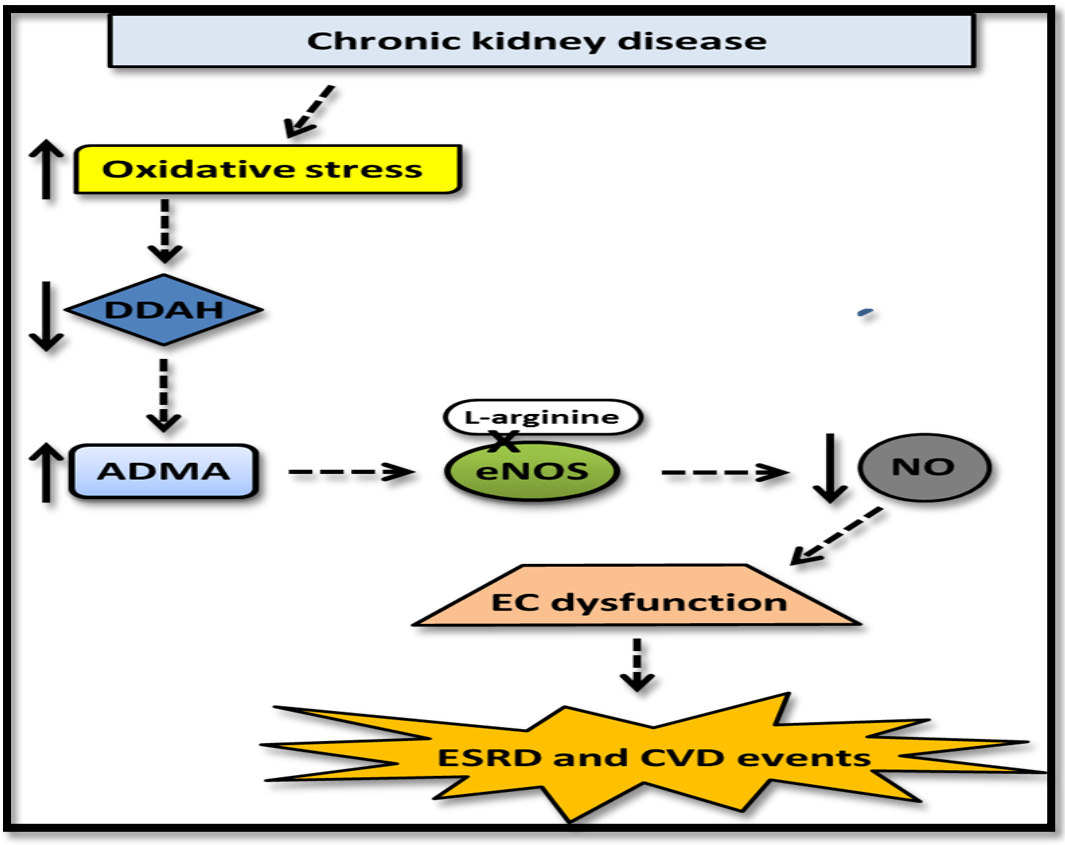


Figure 1.8: Potential mechanism for EC dysfunction in CKD. Inhibition of endothelial eNOS by ADMA occurs via competitive inhibition of L-arginine binding, resulting in reduced NO production and EC dysfunction, CVD events and CKD progression.

Elevated ADMA plasma level has also been reported in various CKD models (Halliwell, 1993). In a model of mouse CKD, high levels of ADMA caused impairment of endothelial function via impairment of eNOS function (Kajimoto *et al*., 2012). This was confirmed by Kajimoto *et al* who used rat model of CKD and showed that, treatment with intravenous DDAH 1 reduced circulating plasma levels of ADMA and oxidative stress, which in turn release the inhibition of eNOS, and consequently reversing CKD-induced EC dysfunction (Pacurari *et al*., 2013).

1.5. Antioxidant treatment

Levels of ROS are maintained by the balance between ROS-generating mechanisms and the efficiency of antioxidant enzymes. Antioxidants are divided into two major groups, enzymatic section 1.2.3, and non-enzymatic antioxidants. Non-enzymatic antioxidants treatment for instance, vitamin C and NAC, have been extensively used both *in vivo* and *in vitro* studies of CKD and CVD, and are designed to reduce ROS and the associated vasculopathy (Kerkeni *et al.*, 2006; Takacs *et al.*, 2001). Because the level of oxidative stress is high in patients with CKD particularly those on dialysis (Jun *et al*., 2012), antioxidant treatment may potentially offer benefits for these patients, and this section will discuss the available evidence regarding the effect of non-enzymatic antioxidants in modifying oxidative stress in CKD patients.

1.5.1. Vitamin C (ascorbic acid)

Vitamin C/ascorbic acid is water soluble and represents one of the most essential antioxidants, and can be administrated orally or intravenously (Rahman, 2007). Doses at 100-200 mg/day of oral vitamin C or 300-500 mg thrice weekly of intravenous vitamin C are considered adequate and safe in HD patients (Kalantar-Zadeh *et al*., 2005; Zhang *et al*., 2013). In CKD and HD patients, 60-100 mg per day of vitamin C are recommended clinically and suggested to exert positive effects by inhibition of lipid peroxidation and decreasing endothelial damage (Biniaz *et al*., 2014). However, this recommendation might not be ideal since on a single dialysis, patients can lose over 100 mg of vitamin C, subsequently causing deficiency of vitamin C (Zhang and Zuo, 2014). *In vivo* oral vitamin C at doses greater than 500 mg

per day showed significant improvement of endothelial function in patients with coronary and peripheral vascular disease as assessed by flow-mediated dilation (FMD), forearm blood flow (FBF), and pulse wave analysis (PWA) (Ashor *et al*., 2014). In addition, vitamin C suppressed endothelial apoptosis mediated by inflammatory cytokines and oxLDL and, therefore, may prevent atherogenesis (Mallat *et al*., 2000). Vitamin C enhances NO synthesis in ECs and *in vivo* it has shown sustained beneficial effects on endothelial-derived NO-dependent flow-mediated dilation (Raitakari *et al*., 2000). However, vitamin C at high concentrations also can act as a pro-oxidant and produce hydroxyl free radicals by participating in the Fenton reaction as shown in the equation below (Cai *et al*., 2001; Zhang *et al*., 2014), causing severe side effects such as elevated generation of oxidative stress when used in the presence of IV iron preparations in CKD patients (Sturm *et al*., 2005). Moreover, because vitamin C is a reducing agent it therefore has the capacity to release iron from ferritin and activate iron from the reticuloendothelial system to transferrin, this in turn leads to increased iron availability and may prevent tissue iron overload further increasing oxidative stress (Sturm *et al*., 2005).

Reaction 1: Ascorbate ($AscH^-$) + Fe^{3+} → Fe^{2+} + Ascorbate radical (Asc^-) + H^+

Reaction 2: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$

1.5.2. *N*-acetyl Cysteine (NAC)

NAC could be derived from food or produced within the human body from cysteine and is rapidly metabolized to intracellular glutathione, which acts as a powerful antioxidant in the body (Atmaca, 2004). Glutathione is synthesised in the body via three amino acids, glutamic acid, glycine and cysteine. Both glutamic acid and glycine are plentiful in cells and therefore the availability of Cys controls glutathione production (Atmaca, 2004). In clinical practice, NAC can be orally administered at dose 600 to 1200 mg twice daily where GFR is less than 60 ml/min per 1.73 m^2 (Rehman *et al,* 2008). NAC has been shown to inhibit NF-κB activation in HUVEC cultured with plasma from women with severe preeclampsia (Takacs *et al.*, 2001). The effect was shown to be through scavenging ROS which are known to activate NF-κB and also block the expression of ICAM-1 mediated by NF-κB. Furthermore, NAC was shown to reduce H_2O_2-induced p38 MAPK activation in lung microvascular EC (Peter *et al.*, 2003). NAC has been shown to be effective in reducing several of cardiovascular events and oxidative stress in HD patients (Tepel *et al*., 2007; Coombes and Fassett, 2012). Following exposure of human proximal tubule epithelial cell line to H_2O_2, NAC decreased lipid peroxidation and maintained the mitochondrial membrane potential, thus preventing apoptosis (Ye *et al*., 2010). In diabetic mouse model of CKD, NAC also reduced level of kidney MDA (Ribeiro *et al*., 2011). In addition, NAC has been previously shown to decrease the plasma level of ADMA (Tepel *et al*., 2003). Furthermore, administration of NAC at 1200 mg twice a week for 15 days, significantly improves renal function in HD patients (Feldman *et al*., 2012). *In vivo* study by Swarnalatha *et al* (2010) showed that

treatment with NAC (600 mg twice daily for 10 days) prior to IV iron therapy with IV iron sucrose reduced EC dysfunction and MDA as a marker of oxidative stress in CKD patients.

1.6. Iron

1.6.1. Iron function and metabolism

Iron is vital for life and an essential trace metal required for almost all living organisms, participating in a wide variety of metabolic and physiological processes in every living cell these include, DNA synthesis, electron transfer reactions, gene regulation, binding and transport of oxygen, and regulation of cell growth and differentiation (Andrews, 1999). In addition, iron is considered as an essential component of many proteins and enzymes that maintain good health. Iron is also essential for expression of protein kinase C-β, a member of the protein kinase C family of intracellular signalling pathways required for cell growth and differentiation (Boldt, 1999). Furthermore, several enzymes including NOS, oxidases, cytochromes, catalases and peroxidises, which are critical in many basic cellular process like DNA and RNA synthesis, and also contribute to the development of a number of diseases contain iron as a key component of these enzymes (Boldt, 1999). The total serum iron levels in the body for adults who ingest a diet free of iron are between 3-4 g (Weinberg, 1990), and its level in the body must be tightly regulated because excessive iron leads to tissue damage.

Roughly 60 to 70% of total body iron is bound to as well as being component of Hb which is circulating in the RBCs (Conrad *et al*., 1999). Another 10% is a constituent of myoglobin, cytochromes, and other iron-containing enzymes

amounting to no more than 0.4-0.8 mg of iron. In healthy individual, the remaining 20 to 30% is bound to the iron storage proteins, ferritin and hemosiderin (Cheng and Li, 2007). Although only 0.1% (approximately 4 mg) of the total body iron is transported in plasma complexed with transferrin, it is the most significant iron pool and has the highest turnover. The turnover of the iron bound to transferrin is about 25 mg/day. 80% of transferrin bound-iron is transported to bone marrow for the synthesis of the Hb in developing erythroid cells (Conrad *et al*., 1999). From these sites, immature RBCs or reticulocytes are released into the circulation and within one day, they develop into mature RBCs (Bottomley *et al*., 1995). Hb is the primary protein in RBCs, transports oxygen form the lung to the tissues and transport back the carbon dioxide to the lung. Hence, iron plays a crucial role in proteins involved in oxygen transport and any deficiency in iron can limit oxygen delivery to cells which results in fatigue and decreases immunity (Andrews, 1999).

1.6.2. Iron chemistry and its role in the human body

1.6.2.1. Iron and oxidative stress

Iron is required for diverse cellular functions. The biological importance of iron is in its chemistry. Iron occurs in oxidation states Fe^{2+} and Fe^{3+}, and is capable of accepting and donating electrons readily. This capacity makes it an important component of molecules that bind to oxygen such as haemoglobin and myoglobin (McCord *et al*., 1998). However, iron is also biochemically dangerous and toxic when present in excess as a result of the formation of free radicals which can damage the cells (Lee *et al*., 2006a).

It is believed that high levels of iron generate oxidative stress due to an increase in the steady state of ROS concentration, both H_2O_2 and superoxide anion raise the toxicity from their iron-dependent transformation into the highly reactive OH· and this can lead to severe damage to proteins, membrane and DNA (Halliwell *et al*, 1984). Under normal conditions, this threat is reduced by the fine regulation of iron metabolism, which prevents the production of ROS. However, under certain pathological conditions, the metabolism of both iron and superoxide are clearly interactive. Each can exacerbate the toxicity of the other (Lipinski and Drapier, 1997). Several studies have demonstrated that, mutations in antioxidant SOD and ferric uptake regulator (fur), which is a protein responsible for coordinating the expression of iron uptake and storage function may lead to excess level of superoxide and iron overload (Iolascon *et al*., 2009). Through these conditions, redox active iron can participate in stimulating lipid peroxidation and the formation of OH· canalization with subsequent tissue damage (Iolascon *et al*., 2009).

Free iron plays a crucial role in oxidative stress (Gutteridge and Halliwell, 1994). Free iron is controlled by a number of automatic mechanisms in the body, which is immediately chelated in cells by compounds such as citrate or ADP and renders it non-toxic or inactive (Prousek, 2007). This unbound or free iron could generate high reactive OH· by participating in the Haber-Weiss reaction, a reaction where OH· generated from an interaction between H_2O_2 and superoxide and catalysed by iron as described below (Prousek, 2007). In the first step of this reaction superoxide anion reduces Fe^{3+} ion to Fe^{2+}.

$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2$$

The second step of the reaction is the Fenton reaction, where the H_2O_2 is reduced to form hydroxyl radical and hydroxyl ion.

$$Fe^{2+} + H_2O_2 \text{ ----> } Fe^{3+} + OH\cdot + OH^-$$

Net reaction

$$O_2^{\cdot-} + H_2O_2 \rightarrow OH\cdot + OH^- + O_2$$

$OH\cdot$ is highly reactive with a half-life of approximately 10^{-9} seconds and has the capacity to abstract a hydrogen atom from polyunsaturated fatty acids (LH) to initiate lipid peroxidation, once lipid hydroperoxide (LOOH) accumulate. An additional lipid peroxidation may be directly initiated by the unbound iron, which will destroy membrane structure and function (Nelson and McCord, 1998).

.

$$OH^\circ + LH \rightarrow H_2O + L^\circ$$

$$L^\circ + LH \rightarrow LOO^\circ$$

$$LOO^\circ + LH \rightarrow LOOH + L^\circ$$

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^\circ + OH^-$$

OH^- can damage lipids, proteins, DNA, sugars, and generally all organic molecules. Hence, high amounts of unbound iron play a crucial role in oxidative stress and can have deleterious effects, especially in case of

overproduction of superoxide, like during inflammatory reactions (Kakhlon and Cabantchik, 2002).

1.6.3. Iron therapy

1.6.3.1. Intravenous iron preparations and CKD

Anaemia is considered one of the major co-morbidities linked to patients with CKD on HD (Besarab, 1999). There are several causes of anaemia in CKD patients, such as poor nutrition, frequent blood loss, vitamin deficiencies (B12 and folate) and inflammation (Koshy *et al*., 2008). However, the primary cause is an inadequate quantity of endogenous EPO. In addition, the vast majority of patients with CKD can already have iron deficiency which is also a common cause of anaemia as a result of low protein diet or gastrointestinal bleeding (Eschbach and Adamson, 1985). EPO is a glycoprotein hormone produced by the peritubular capillary cells in the kidney. It promotes the formation of red blood cells in the bone marrow. Therefore, optimal management of anaemia associated with CKD can be efficiently corrected by recombinant human erythropoietin (rhEPO), widely and routinely used instead of transfusion of blood, which was formerly used (Nissenson, 1991). In CKD patients, in order to avoid iron deficiency, maintain adequate iron stores (serum ferritin > 100 µg/L or TSAT > 20%) and support erythropoiesis, iron replacement therapy should be administered therefore reducing consumption of the highly cost rhEPO and to achieving haemoglobin target level in order to manage the anaemia (Hasegawa *et al*., 2011). Iron can be given either orally or intravenously, and because oral iron is adequate for the treatment of many iron-deficient patients poorly absorbed in CKD patients

due to gastrointestinal upset (diarrhoea and constipation) or due to the inflammation associated with these patients (Spinowitz *et al*., 2008). Therefore, IV iron is better choice than oral iron and considered as the key component of anaemia management for CKD patients. IV iron is administered frequently (≤200 mg), needing from 5 to 8 visits for the administration of 1 g or it can be administered as a large dose depending on IV iron type (Silverberg *et al*., 1999; Van Wyck *et al*., 2005; Spinowitz *et al*., 2008). IV iron was first introduced for human use in the early 20th century (Heath *et al*., 1932; Garneata, 2008). There are a variety of IV iron products used in treatment of iron deficiency anaemia in CKD such as low molecular weight iron dextran, ferric gluconate, iron sucrose, ferric carboxymaltose (FCM) and ferric chloride hexahydrate (Moore *et al*., 2011). Iron dextran was commonly used from 1950 to 2000, however has subsequently been largely replaced worldwide by ferric gluconate and iron sucrose (Wang *et al*., 2008). IV iron preparations have formulations that consist of an iron core surrounded by a stabilized carbohydrate shell to encapsulate the bioactive iron; this was because of the instability of the compound, permitting iron to dissociate in the circulation causing toxicity (Danielson, 2004). Each one ml of IV iron sucrose solution contains 20 mg of iron as iron sucrose (iron (III)-hydroxide sucrose complex), where one mL of IV FCM solution contains 50 mg of iron as ferric carboxymaltose. These types of IV iron preparations all differ in core size and identity of the shell, which determine the pharmacological properties of the agent such as stability and iron release (Danielson, 2004). In addition, pharmacokinetic properties vary between these compounds, where IV iron sucrose has the lowest molecular weight

(140 KDa), shortest half-life (6hr) and lowest stability compared with IV FCM which has higher molecular weight of (233 KDa), half-life of (7-12hr) and is more stable (Jahn *et al*., 2011; Geisser and Burckhardt, 2011; Nordfjeld *et al*., 2012). All these differences suggest that IV FCM may have a safer profile and may induce less toxicity than IV iron sucrose (Funk *et al*., 2010; Toblli *et al*., 2011; Prats *et al*., 2013). Iron dextran can be administered as a single dose, but this requires administration over a period of 4 to 6 hr, but has been associated with hypersensitivity reactions that have limited its use (Bailie *et al*., 2005; Gisbert and Gomollón, 2008). IV iron sucrose is given as 1000 mg in divided doses, as a slow push injection or 15 to 30 min infusion in doses of 100-200 mg, which require multiple outpatient visits and repeated intravenous access. IV FCM can be administered as a large replenishment dose (1000-1500 mg of iron) over a short infusion in 15-30 min; however, only doses up to 1000 mg are currently approved (Lyseng-Williamson and Keating, 2009). It has been documented that parenteral iron administration is efficient and safe (Chertow *et al*., 2006). However, because of iron's potent oxidant activity, concerns have been raised about parenteral iron leading to over saturation of transferrin, producing iron overload and potentially increasing oxidative stress which is already a factor in CKD patients (Deicher and Horl, 2002). Following injection, IV iron supplementations are initially processed in the reticuloendothelial system (RES) of the liver and spleen but large amounts of these compounds are distributed to RES of the bone marrow, where phagocytes liberate iron to be stored in ferritin or released back to the circulation as transferrin-bound iron (Beshara *et al*., 1999; Beshara *et al*., 2003). Large doses of IV iron can exceed storage capacity

leading to non-transferrin-bound-iron (NTBI) being released into the circulation. In addition, some iron can directly bind to transferrin without passing through RES as shown in Figure 1.9; this unbound iron via the Fenton reaction can produce the potent hydroxyl radical (Kalyanaraman, 2013), as described in section 1.2.3. All these factors are believed to increase oxidative stress, thereby worsen the cardiovascular outcome of CKD patients, leading to EC dysfunction and inflammation as shown in Figure 1.9. Furthermore, *in vivo and in vitro* studies have shown that IV iron administration increases oxidative stress biologic markers; iron sucrose increases oxidative stress markers of lipid peroxidation such as MDA and free iron in dialysis patients. Iron sucrose can also be associated with EC injury and dysfunction, promoting apoptosis and inhibiting proliferation in cultured ECs (Kartikasari *et al*., 2006). Zager *et al* found that all the three IV iron preparations mentioned above led to some degree of lipid peroxidation (Zager *et al*., 2002). In a study in 2004 on 20 patients at stages 3-4 of CKD, who were injected with 100 mg IV iron sucrose over 5 min, increased plasma and urine levels of MDA occurred after 15-30 min of administration, and returned to baseline after 24hr (Agarwal *et al*., 2004). Leehey *et al* found that IV administration of ferric gluconate to CKD patients, increases oxidative stress, assessed by increased level of plasma and urine MDA (Leehey *et al*., 2005). In 2007, another study was carried out to investigate the effect of IV iron administrations on markers of oxidative stress. This study, involved 12 patients undergoing HD, who each received 100 mg/ml IV iron sucrose, iron dextran or ferric gluconate consecutively with 14 days washout stage between each IV iron compound administration. It was found that IV iron

sucrose and ferric gluconate were associated with greater NTBI levels compared with iron dextran. In addition, all three compounds induced oxidative stress as reflected by increased level of MDA (Pai *et al*., 2007).

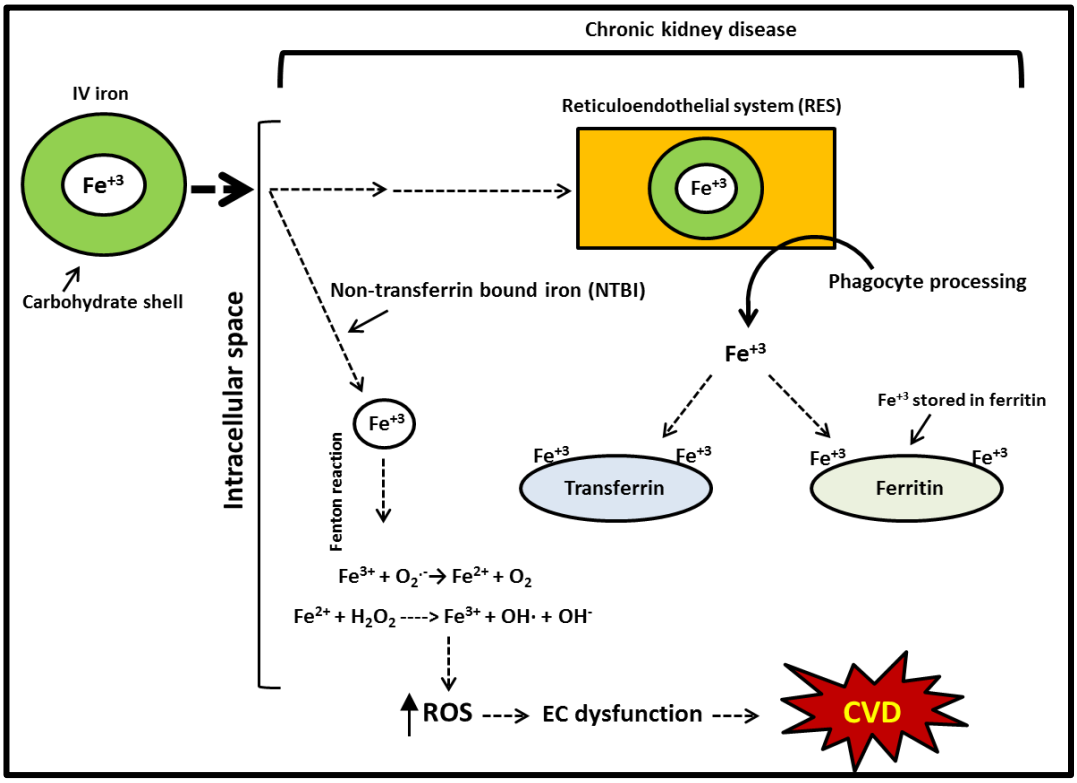


Figure 1.9: Metabolism of IV iron preparation. Once administered IV iron is processed in the RES, where phagocytes liberate iron to be stored in ferritin or released back to the circulation as transferrin-bound iron. IV formulations can deliver a small amount of iron directly to transferrin without being processed in the RES. There is concern that oversaturation of transferrin results in increased extracellular iron bound to negatively charged compounds and is accessible for generation of free radicals via the Fenton/Haber–Weiss reactions. This unbound iron or NTBI via the Fenton reaction can produce the hydroxyl radical, resulting in EC dysfunction and subsequently CVD in CKD patients.

1.7. Apoptosis

Apoptosis or programmed cell death was coined by Kerr *et al*. in 1972, and can be distinguished from necrosis through different morphological and biological features such as, cell shrinkage, membrane blebbing, nuclear and cell fragmentation as shown in Figure 1.10 (Ding *et al*., 2007). Apoptosis is an essential biological and tightly regulated process for normal development to maintain cell populations in tissues and maintenance of homeostasis also occurs as a protective mechanism such as in immune reactions or when cells are damaged by disease including cancer and CVD (Norbury and Hickson, 2001). A key feature that differentiates apoptosis and necrosis is that apoptosis does not induces an inflammatory reaction (Rock and Kono, 2008). In mammals, initiation and regulation of apoptosis can be through one of two major signalling pathways the intrinsic pathway, which depends on the participation of mitochondria and is mediated by Bcl-2 family members, and the extrinsic pathway which occurs through death-receptor interactions, principally those involving the tumour necrosis factor-receptor (TNF-R) family, including TNF receptor-1 or the Fas receptor (Zimmermann and Green 2001). These two pathways are responsible for the activation of cysteine protease enzymes known as caspases. There are three types of caspase and initially all are present as inactive pro-enzymes that require dimerization including, initiator caspases (2, 8, 10 and 9), effector caspases (3, 6 and 7) and inflammatory caspases (1, 4 and 5) (Cohen, 1997; Strasser *et al*., 2000).

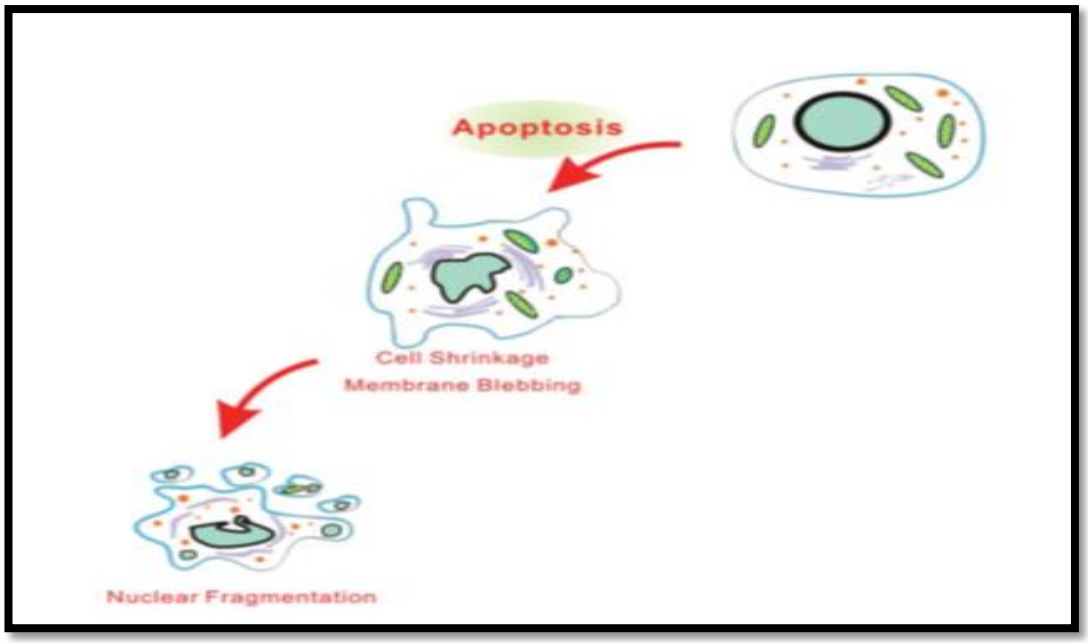


Figure 1.10: Image shows different morphological and biological features of apoptosis such as, cell shrinkage, membrane blebbing, nuclear and cell fragmentation.

1.7.1. Extrinsic pathway apoptosis

In the extrinsic pathway, the death receptor Fas recruits Fas-associated death domain (FADD) to the death-inducing signalling complex (DISC) through interaction with death effector domain (DED) of pro-caspase-8, which in turn result in its autocatalytic activation (Ashkenazi, 2002). The initiator caspase-8 activates further caspases (including the effector caspases-3, 6 and 7), which are critical in cell death (Yang *et al*., 2005) as shown in Figure 1.11.

1.7.2. Intrinsic pathway apoptosis

The mitochondrial apoptotic pathway is triggered by numerous stimuli including DNA damage, cytotoxic drug, oxidative stress and pro-apoptotic signal-transducing molecules which act on mitochondria to induce outer

mitochondrial membrane permeabilization (Chipuk and Green, 2008; Muñoz-Pinedo, 2012). This permeabilization is regulated by pro-apoptotic proteins from the B cell lymphoma-2 (Bcl-2) family such as Bax (Fulda and Debatin, 2006). Upon disruption of the outer mitochondrial membrane, proteins normally found in the space between the inner and outer mitochondrial membranes are released, including cytochrome *c* (Loreto *et al*., 2014). When released from mitochondria into the cytosol, the cytochrome *c* promotes the assembly of apoptosome (Rowinsky, 2005; Loreto *et al*., 2011). The apoptosome is a multimeric protein complex involving apoptotic protease activating factor-1 (Apaf-1) where the cytochrome *c* binds to Apaf-1 and caspase-9, and forms a complex which becomes activated in the presence of ATP or Deoxyadenosine triphosphate (dATP) (Yu *et al*., 2005). The activated caspase-9 subsequently cleavages other downstream caspases like caspase-3, which result in cell apoptosis (Li and Yuan, 2008; Tan *et al*., 2014).

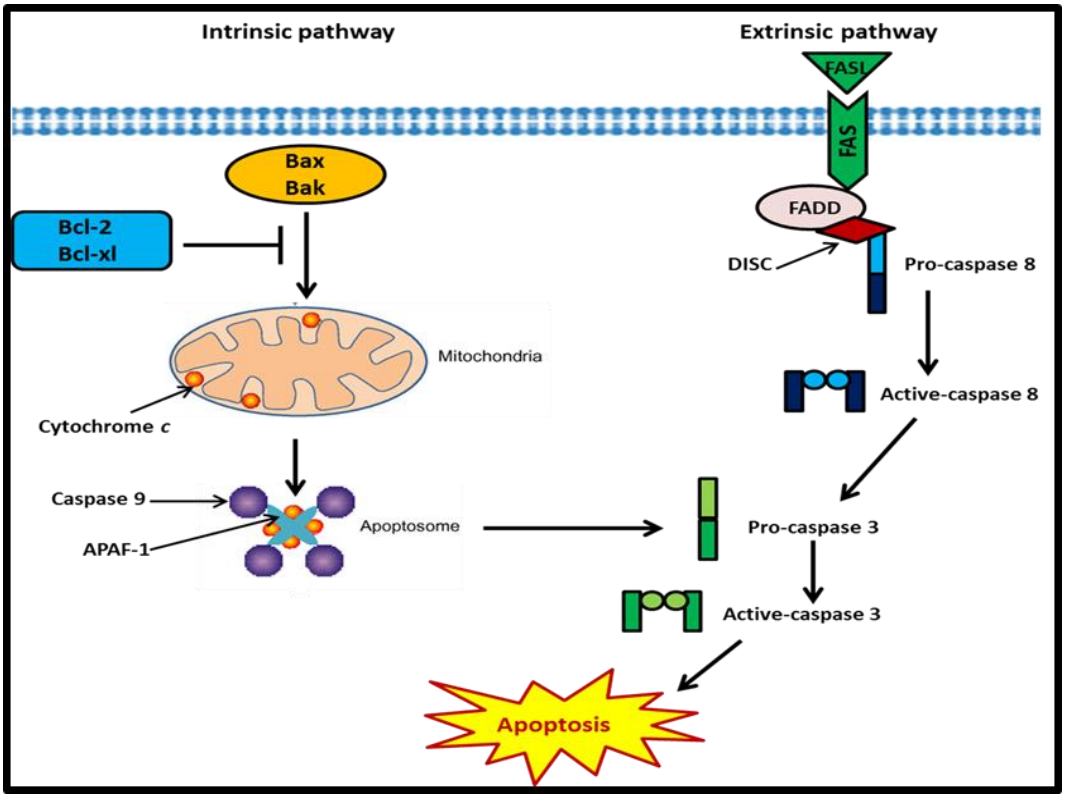


Figure 1.11: Representative image shows the apoptosis pathways (intrinsic and extrinsic).

1.7.3. Bcl-2 Family proteins

Bcl-2 was first identified as a gene product that causes resistance to apoptosis in lymphocytes (Bakhshi *et al*., 1987). The Bcl-2 family proteins are key regulators of apoptosis and can be divided into three main subfamilies based on their function and the number of BCL-2 homology (BH) domains. Family members include, anti-apoptotic proteins (Bcl-2, Bcl-xL,A1 and Mcl-1), which are characterized by the BH1-4 domains, and two pro-apoptotic protein groups (Bax and Bak), and (Bim, Puma, Noxa, Bid, Bad, Bik, Bmf, Hrk), which are characterized by the presence of BH1-3 domains and BH3-only group (containing only the BH3 domain) (Wang *et al*., 1996; Brunelle and Letai, 2009) as shown in Figure 1.12. In normal viable cells, the

majority of Bax exists as a monomer in the cytosol or attached to the outer mitochondrial membrane, and translocate to the mitochondria in response to apoptotic signals (Wolter *et al*., 1997). Bax and Bcl-2 play an essential role in regulating the changes in mitochondrial outer membrane permeability (MOMP) (Youle and Strasser, 2008). The anti-apoptotic Bcl-2 promotes cell survival by preventing changes in MOMP by neutralizing the activity of both pro-apoptotic members BH3 only protein and multi domain members (Youle and Strasser, 2008), while activation of pro-apoptotic protein Bax and Bak can strongly influence the progression of cells towards death via apoptosis through the release of apoptogenic factors, such as cytochrome *c* and Smac from mitochondrial intermembrane space (IMS) into the cytosol (Wei *et al*., 2001; Wang, 2001). Cytochrome *c* is a water soluble protein and considered as the main mediator of apoptosis, which can be inhibited by overexpression of Bcl-2 protein (Yang *et al*., 1997; Pellegrini and Scorrano, 2007). Once released, cytochrome *c* promotes the formation of the apoptosome that includes pro-caspase-9 and its adaptor Apaf-1 (apoptotic protease-activating factor-1), transforming pro-caspase-9 into its active form, which in turns proteolytically activates the effector caspases (3, 6 and 7) to initiate the execution of apoptosis (Degterev *et al*., 2003).

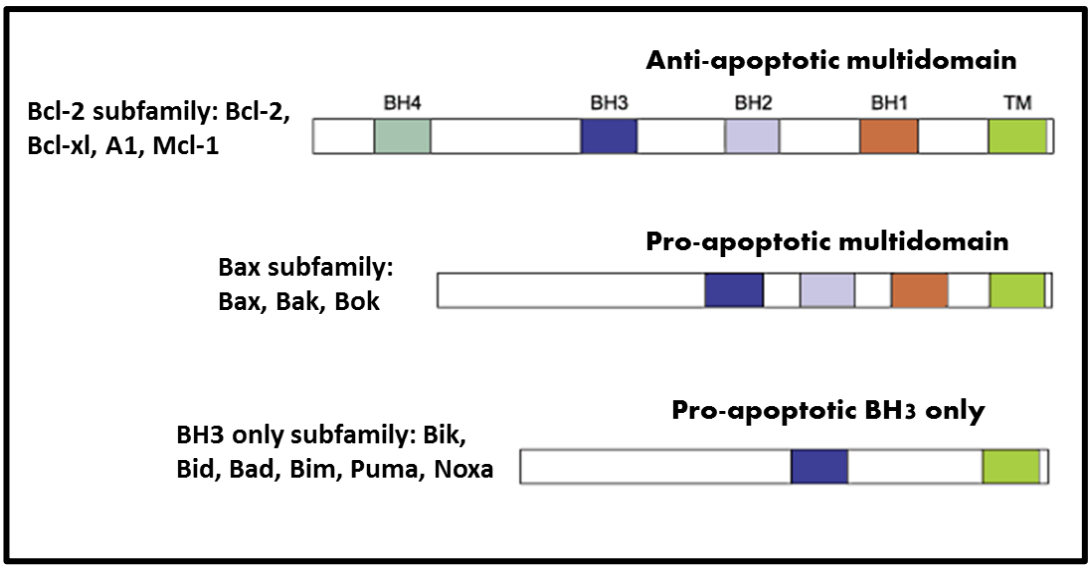


Figure 1.12: The Bcl-2 protein family members. Anti-apoptotic Bcl-2-family members contain all four BH domains. Pro-apoptotic Bcl-2-family members can be separated into multi-domain or BH3-only proteins. Some pro-apoptotic proteins (BH3-only proteins) do not have a transmembrane Domain (TM), such as Bid, Puma, Bad and Noxa.

1.8. Mitogen activated protein kinase (MAP kinase)

Mitogen activated protein kinases (MAPKs), are a family of serine-threonine kinases that mediate important cellular functions and responses including proliferation, differentiation, development, transcription, stress response, and apoptosis (Plotnikov *et al*., 2011). They mediate cellular responses to external stimuli, including soluble factors such as, growth factors, hormones and cytokines (Barr and Bogoyevitch, 2001). MAPKs require dual threonine and tyrosine phosphorylation in an activation loop (Thr-Xaa-Tyr motif), in which MAPKK Kinase (MAPKKK or MAP3K) phosphorylates and activate MAPKK or (MAP2K). This MAPKK in turn phosphorylates and activates MAPK, which phosphorylate and activate their specific target proteins both

cytosolic proteins and transcription factors such as C-myc (Huang and Ferrell, 1996; Barr and Bogoyevitch, 2001; Roux and Blenis, 2004). The MAPK signalling cascades are often initiated by receptor-mediated activation of members of the small monomeric G protein family, such as Ras, Rac or Rho (Loirand *et al*., 2013). The MAP kinase pathway consists of four families, which are referred to as extracellular signal-regulated kinases (ERKs) consisting of two proteins: ERK1 and ERK2, p38 MAPK: p38α, p38β, p38γ and p38δ MAPK, Jun-N-terminal kinases (JNKs): JNK1, JNK2 and JNK3 (also known as stress-activated protein kinases, SAPKs). There is also ERK5 as shown in Figure 1.13 (Sebolt-Leopoldm and Herrera, 2004; Wagner and Nebreda, 2009). In mammals, there are seven MAPKKs, which have some specificity towards MAPKs as shown in Figure 1.13 (Rose *et al*., 2010). These MAPKKs consist of, MEK1 and MEK2 which activate ERK 1 and ERK2, MEK5 for activation of ERK5, MKK4 and MKK7 activate JNKs, and MKK3 and MKK6 to activate p38 MAPKs as shown in Figure 1.13 (Rose *et al*., 2010). In addition, there are at least twenty MAPKKK, of which six regulate the ERK1 and 2 pathways, twelve regulate the JNK pathway and ten MAP3Ks regulate the p38 MAPK pathway as shown in Figure 1.13 (Cargnello and Roux, 2011).

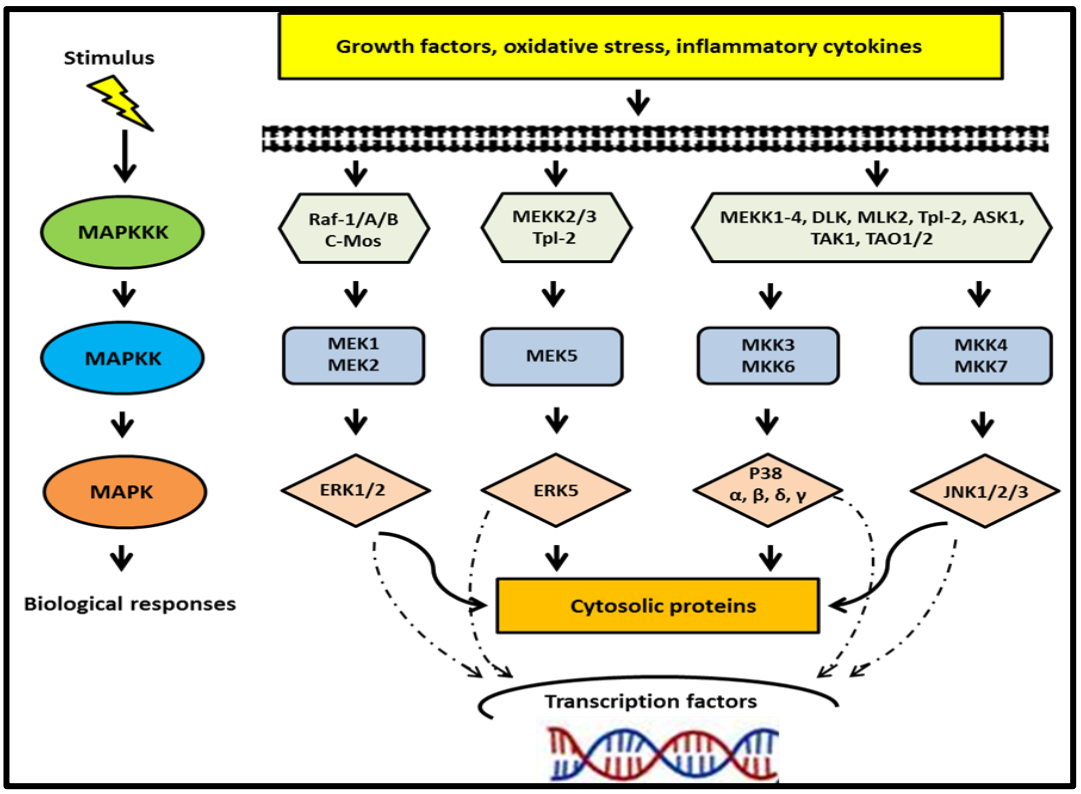


Figure 1.13: The Schematic of MAPK signalling pathways. Activation pathway of all MAPK family members is composed of three consecutive kinases (MAPKKK, MAPKK, and MAPK).

ERK1 and ERK2 pathway can be activated in response to growth factors, cytokines, certain stresses, ligands for small G protein-coupled receptors (GPCRs), and are involved in cell proliferation and survival (Svensson *et al.,* 2011). JNK and p38 MAPK can be activated in response to a number of exogenous stimuli like growth factor deprivation, pro-inflammatory cytokines such as TNF-α, and have been shown to be implicated in apoptosis, proliferation and survival, depending on the stimuli and cellular conditions (Junttila *et al*., 2008; Schutters and Reutelingsperger, 2010). P38 MAPK will be discussed in more detailed in section 1.8.1.

1.8.1. Role of p38 MAP kinase in apoptosis

There are three isoforms expressed in ECs, p38α, p38β and p38δ (Cuenda and Rousseau, 2007). Isoforms p38α and p38β are ubiquitously expressed at significant levels in ECs and they are sensitive to pharmacological inhibitors such as SB203580, which act as specific inhibitors of p38 MAPK signalling pathway (Kumar *et al*, 1997; Porras *et al*., 2004), p38α is also the most abundant p38 MAPK family member, while p38γ and p38δ are differentially expressed depending on the tissue (Porras *et al*., 2004). P38 MAPK signalling pathways are activated by phosphorylation on both tyrosine (Tyr182) and threonine (Thr180) residue, which is mediated by the upstream activation of MEK3 and MEK6 (Derijard *et al*., 1995; Banh and Hales, 2013). In addition, MAP3Ks shown to trigger the activation of p38 MAPK pathway include ASK1 (apoptosis signal kinase 1), TAK1 (transforming growth factor β-activated kinase 1) and TPL2 (tumour progression loci 2) as shown in Figure 1.13. P38 MAPKs are activated in response to different inflammatory cytokines, hormones, stress factors, ligands that activate GPCRs and physical stresses such as, osmotic and heat shock leading to the regulation of cellular functions such as proliferation, differentiation and survival (Pearson *et al*., 2001). P38 MAPK is also activated in response to elevated oxidative stress and this activation was shown to be associated with apoptosis (Callsen and Brune, 1999). Role of p38 MAPK in the regulation of apoptosis is dependent on the cell type and stimuli. In neuronal cells, p38α MAPK was shown to mediate apoptosis (Wang *et al*., 1998), and in cardiac cells upon stimulation with TNF-α (Ghatan *et al*., 2000).

P38 MAPK was shown to mediate Bcl-2 protein phosphorylation in growth factor-starved memory B lymphocytes, which caused apoptosis (Torcia *et al*., 2001). In addition, up-regulation of both Bax protein expression and Bax mRNA has been reported to be mediated by p38 MAPK in a cardiomyocyte derived cell line, leading to apoptosis (Porras *et al*., 2004). Furthermore, in a study by Grethe *et al* (2004) showed that treating an endothelial cell line (EA.hy926) with TNF-α induced phosphorylation of p38 MAPK which in turn led to the activation of caspase 3, and treatment with p38 MAPK inhibitor (SB203580) caused 50% reduction of TNF-induced activation of caspase-3 and cell death, indicating that p38 MAPK play an important role in the apoptosis of ECs. As overproduction of ROS plays major role in the initiation and progression of CVD, its role as a second messenger to activate p38 MAPK and its associated downstream transcription factors such as NF-κB and AP-1, which then can regulate the expression of numerous pro-inflammatory genes, results in cell injury (Sun *et al*., 1996; Dröge, 2002). Accumulation of intracellular ROS disrupt the mitochondrial membrane potential, release of cytochrome *c* with subsequent activation of the caspase cascade, and, ultimately, leads to apoptosis (Jabs, 1999; Jing *et al*., 1999). ROS such as H_2O_2 was shown to induce apoptosis of ECs via activation of p38 MAPK (Natarajan *et al*., 2001). Recently generation of ROS in EBV-transformed B cells was shown to activate p38 MAPK, resulting in up-regulation of Bax and activation of caspase-9 and caspase-3, and subsequent apoptosis (Park *et al*., 2014).

1.9. AIMS

As has been reviewed in the introduction, vascular damage in CKD is a major problem. The reliance on IV iron treatment means it is important to investigate the mechanisms and extent by which different iron treatments induce activation and damage to EC, leading to dysfunction. Thus, fully understanding the mechanisms leading to vascular damage by IV iron preparations can be beneficial in designing treatment regimens to reduce development, progression and early morbidity and mortality of CVD in patients with CKD. Due to lack of availability of ECs from CKD patients, clinical iron preparations will be used *in vitro* with primary EC from healthy sources (HUVEC). Treatment of healthy EC with the clinically IV iron preparations used for CKD patients will be critical to understand the mechanisms that lead to EC dysfunction and which can then be extrapolated to effects in the kidney of patients with CKD. The project has a number of objectives:

- Study the ability of IV iron sucrose or IV FCM to alter the growth characteristics, morphology and viability of HUVECs.
- Assessment of the extent to which each IV iron preparations induce endogenous ROS generation in HUVEC by the measurement of intracellular superoxide anions and total intracellular ROS production in HUVEC with the aim of correlating with differential EC damage and CV risk in CKD patients.
- Determine extent of functional changes seen in the EC in response to IV iron sucrose or IV FCM exposure. Both EC activation by the

assessment of endothelial adhesion molecule expression (ICAM-1 and VCAM-1) and apoptosis will be quantified. This *in vitro* data links the role of ROS to functional changes in EC which importantly can be correlated with increased CVD, the primary cause of death in patients with CKD.

- Importantly the *in vitro* system allows direct assessment of the mechanisms important in driving these functional changes, something difficult to assess *in vivo* and signalling pathways with a role in apoptosis such as Bcl-2/Bax and p38 MAP kinase will be identified.
- Evaluation of the role of IV iron compounds in EC apoptosis, which may be a promoter of atherosclerosis. Specifically of interest is p38 MAPK pathway and associated proteins, such as Bcl-2 and Bax.
- Investigation of the benefit of treatment with antioxidants such as NAC in reducing oxidative stress generated by IV iron preparations and their downstream ability to cause EC dysfunction, therefore giving an indication of potential therapeutic strategies for preventing or decreasing EC dysfunction/damage and CVD in patients with CKD.

CHAPTER 2

THE EFFECT OF INTRAVENOUS IRON PREPARATIONS ON GROWTH OF HUMAN ENDOTHELIAL CELLS

2.1. INTRODUCTION

CKD is a worldwide health concern, affecting approximately 5-7% of world population (Couser *et al*., 2011) and over 13% of the western population (Coresh *et al*., 2007). CKD is recognised as an important risk factor for CVD development (Moe *et al*., 2003). Particularly, patients in early stages of this complex disease have a higher risk of death due to CVD, than they are to progress to ESRD or renal transplantation (Schiffrin *et al*., 2007). In addition, CVD is considered as the main cause of death among CKD patients, with the rate of CVD being twice as common compared with the general population (Collins *et al*., 2003). The risk of death from CVD in dialysis patients is 9% per year of haemodialysis (Coen *et al*., 2007), and 30% of all deaths worldwide among general population (Anderson and Chu, 2007). A study by Keith *et al* (2004), on CKD patients at stages 2, 3 and 4 showed that, death due to CVD occurred before the patients had the opportunity to receive renal replacement therapy. Furthermore, a UK study found that only 4% of individuals progressed to ESRD over a 5.5 year follow-up period, whilst 69% had died at the end of follow-up; the cause of death was cardiovascular in 46% of cases (Drey *et al*., 2003). Atherosclerosis is an inflammatory vascular disease, characterized by the accumulation of lipids, inflammatory cells, and connective tissue within the intima-media layer of the arterial wall (Libby, 2002). Atherosclerosis is frequently associated with CVD and has been recognized as one of the most serious and frequent complications occurring in patients suffering from CKD (Luczak *et al*., 2011). In addition, these patients have a 20 fold higher prevalence of premature arterial atherosclerosis compared with general population (Luczak *et al*., 2011).

Atherosclerosis is considered as the leading cause of ischemic heart disease in dialysis patients (Luczak *et al*., 2011). ECs act as a barrier (the endothelium) between tissue and the blood constituents (Belle *et al*., 1998; Bonetti *et al*., 2003). ECs plays critical role in overall homeostasis by the synthesis of various paracrine substances that regulate vascular tone and permeability, coagulation and fibrinolysis, and inflammatory responses in the vessel wall, including NO, angiotensin II and ROS. ECs also interact with other cell types such as VSMC, platelets, and leukocytes (Pober and Sessa, 2007). Due to these roles anything that causes EC dysfunction could be considered among the primary causes of atherosclerosis (Davignon and Ganz, 2004).

Iron deficiency is found in most CKD patients particularly in patients on haemodialysis, and is a frequent cause of anaemia in these patients (McClellan *et al*., 2002; Yessayan *et al*., 2014). The first recourse is iron supplementation, either oral or parenteral, and since oral iron is poorly absorbed, and is not well tolerated because of adverse gastrointestinal effects (Kulnigg and Gasche, 2006; Rozen-Zvi *et al*., 2008). IV iron preparations were developed to overcome these problems in CKD patients (Bailie *et al*., 2005; Gisbert and Gomollón, 2008). IV iron preparations used clinically have included iron as high or low-molecular-weight iron dextran, ferric gluconate, IV iron sucrose and IV FCM (Kulnigg *et al*., 2008; Dillon *et al*., 2012). These compounds are widely used in the treatment of anaemia by improving serum haemoglobin in CKD patients and to lower the requirement of the high cost erythropoiesis-stimulating agents or rhEPO in these patients (Besarab *et al*., 2000; Dillon *et al*., 2012). However, all of these compounds

despite their differences can lead to saturation of transferrin and, consequently, to elevated significant blood levels of NTBI or free iron due to the rapid release of iron from its complex, particularly if high doses are administered (Kooistra *et al*., 2002; Schaller *et al*., 2005). Functional and structural disintegration of ECs due to impaired NO bioavailability, is a key element in the progression of CKD, and may explain the increased cardiovascular risk in these patients (Fliser *et al*., 2011). EC is frequently observed in CKD and it may be further aggravated by IV iron supplementation (Cross, 2002; Shah and Alam, 2003). There is abundant evidence that IV iron administration, including IV iron sucrose can inhibit EC proliferation and mediate damage of ECs *in vitro* (Zager *et al*., 2002; Kamanna *et al*., 2012) and *in vivo* (Rooyakkers *et al*., 2002; Zheng *et al*., 2006), which is likely to be a key step in accelerated atherogenesis since EC represent the primary site for the initiation of atherosclerosis, which would finally lead to plaque progression and occurrence of atherosclerotic complications (Davignon and Ganz, 2004). In contrast, IV FCM is a new preparation of non-dextran iron which, can be administered as a large replenishment dose over 1000 mg of iron over a short infusion in 30 min. IV FCM has been shown to induce less toxicity and less cellular damage than other IV iron compounds, due to its robust iron-carbohydrate complex stability and pharmacokinetics properties (Funk *et al*., 2010; Toblli *et al*., 2011; Prats *et al*., 2013). However, to our knowledge the effect of this compound on EC proliferation, injury and damage *in vitro* has not been tested. Therefore, two IV iron preparation were chosen in this study, IV iron sucrose as the commonest and agent with most published literature and IV

FCM. The effect of these compounds on *in vitro* growth characteristics, viability and proliferation rate of human ECs was studied.

2.2. MATERIALS AND METHODS

2.2.1. Materials

2.2.1.1. Cell culture

All tissue culture reagents used were of tissue culture grade. All tissue culture plastics used (T75 cm^2) flasks, 96-well plates and 6 well plates were either from Corning Incorporation (Corning, NY 14831, USA) or Becton-Dickinson (Falcon), (Leeds, England UK). Disposable sterile pipettes of volume 5, 10 and 25 ml were from Costar Corporation, Cambridge or from LIP Equipment & Services Ltd, (Shipley, UK). Disposable syringes were purchased from National Health Supplies (Leeds, UK).

Human umbilical vein endothelial cells (HUVEC) were obtained from several sources: Ethical Tissue Bank (University of Bradford, UK), Promocell, Sickingenstrasse, Germany catalogue number C-12250 or from Caltag-Medsystems, Buckingham, UK catalogue number ZHC-2106. HUVEC used throughout all this study, were from a number of individual donors as shown in table 2 .1.

All cell culture plastics used for the culture of HUVEC were coated in 10% (v/v) bovine gelatin in PBS prior to use for at least 30 min and rinsed with PBS prior to addition of cells. All cell culture procedures were performed using aseptic techniques in biological safety cabinet. All culture were

preserved in a humidified in 5% Carbon dioxide (CO_2), 95% humidified air and at 37^0C.

Table 2.1: List of primary HUVEC with catalogue number, source materials, passage number and the providers.

Catalogue number	Source materials	Tissue / Material	Passage number/Comments	Provider
ET-10-3595	1	HUVEC	$1x10^6$ P1/single donor	Ethical Tissue Bank University of Bradford
ET-10-3429	1	HUVEC	$1x10^6$ P1/single donor	Ethical Tissue Bank University of Bradford
ET-11-3738	1	HUVEC	$1x10^6$ P1/single donor	Ethical Tissue Bank University of Bradford
ET-11-3742	1	HUVEC	$1x10^6$ P1/single donor	Ethical Tissue Bank University of Bradford
ET-12-4314	1	HUVEC	$1x10^6$ P1/single donor	Ethical Tissue Bank University of Bradford
ET-12-4739	1	HUVEC	$1x10^6$ P1/single donor	Ethical Tissue Bank University of Bradford
ZHC-2106	5	HUVEC	$5x10^5$ P1/single donors	Caltag Medsystems
C-12250	5	HUVEC	500,000 proliferating cells/single donors	Promocell

2.2.1.2. Reagents

Penicillin/streptomycin (15140-115), trypsin/ethylenediaminetetraacetic acid (EDTA) (E5134), Fungizone (15290-026) and L-glutamine (25030-024) were obtained from Gibco (Paisley, Scotland, UK). Trypan blue (T6146), gelatin (G1393), dimethyl sulfoxide A.C.S spectrophotometric grade (DMSO, D-8779), Fluorescein-conjugated mouse monoclonal antibodies against human

CD31 (PECAM-1) (F8402), paraformaldehyde (P6148), Triton X-100 (T9284) and Thiazolyl Blue Tetrazolium Bromide (MTT) (M2128) were all purchased from Sigma-Aldrich Chemical Company (Poole Dorset, UK). IV iron sucrose (Venofer) 100 mg in 5 ml and IV ferric carboxymaltose (FCM) or (Ferinject) 100 mg in 2 ml (DVF04499) were purchased from Bradford (Hospitals NHS trust, UK). Dulbecco's Phosphate Buffer Saline (DPBS) (SH 3026401) and Lab-Tek™ II Chamber Slide™ System (154534) were purchased from Thermo Scientific (Nottingham, UK). Phenol red-free M199 media (11043-023) was supplied by Invitrogen (Paisley, UK). Endothelial cell growth medium (C-22020) was purchased from Promocell (Sickingenstrasse, Germany). Human large vessel endothelial cell growth medium (ZHM-2953) were obtained from Caltag-Medsystems (Buckingham, UK). TrypLE™ Express enzyme (1X), phenol red free (12604-013) was purchased from Life Technologies Ltd (Paisley, UK). Foetal bovine serum (FR-1090) was obtained from Biosera (East Sussex, UK).

2.2.2. Methods

2.2.2.1. Culture of HUVECs

HUVEC were maintained in endothelial cell growth medium supplemented with (100U/100 mg/ml) penicillin-streptomycin and supplement mix (0.02 ml/ml foetal calf serum and 0.004 ml/ml endothelial growth supplement), L-glutamine (2 mM) and Fungizone (2.5 µg/ml) and cultured at 37^0C in a humid atmosphere containing 5% CO_2 in 95% air. Cells were then grown in endothelial cell growth medium (C-22020) or (ZHM-2953) and were propagated in gelatin coated plastic tissue culture T75 cm^2 flasks. After

reaching confluence, the cells were sub-cultured and reseeded. Subsequent to trypsinization by trypsin/EDTA (0.05%/0.02%), the cells considered to be round in shapes and in suspension. After 24hr the medium was discarded, and cells were washed twice with warm phosphate buffer saline (PBS) to remove cell debris and any dead cells. Upon confluence, HUVEC were passaged at ratio of 1:3 up to passage number 4, as they have been characterized within the lab (Sarkanen *et* al., 2010) to respond to stimuli in a manner consistent with newly isolated endothelium until passage 4. HUVEC which were not able to adapt to culture conditions were discarded. All cell culture plastics (flasks or plates) used for the culture of HUVEC were coated in 10% v/v gelatin in PBS prior to use for at least 30 min and rinsed with PBS prior to addition of cells.

2.2.2.2. Immunocharacterisation of HUVEC

Cells were seeded at a concentration of 2 x 10^5 cells per ml (500 µl/chamber) in chamber slides and cultured at 37^0C in a humid atmosphere containing 5% CO_2 in 95% air. Once HUVEC reached the confluence state, the media was discarded and HUVEC were fixed using 4% w/v paraformaldehyde (in PBS) for 10 min at room temperature. Subsequently, the fixative was discarded and cells were washed thoroughly three times with PBS. Fixed HUVEC were then permeabilized with PBT (0.1% (v/v) Triton X-100 in PBS) for 10 min. the cells were blocked with blocking buffer (1% w/v BSA in PBS) for 90 min and then they were incubated with 200 µl mouse monoclonal anti human CD31 antibodies (1:20) overnight at 4°C. HUVEC from same donors were incubated with PBS and considered as negative control. Cell nuclei were counterstained by Vectashield with 4', 5-Diamidino-2-phenylindole (DAPI).

Images were obtained using an Eclipse TE2000 inverted research microscope (Nikon DS-U1, Tokyo, Japan) version 5.03 and photographed with a cooled Hamamatsu digital camera (Hamamatsu, Japan) using 20x objective. Photos of randomly chosen fields were taken in each experiment using the FITC- filter and DAPI- filter for antibodies-treated and non-treated cells and compared to check the basal level.

2.2.2.3. Expansion of cultures

After the cells become confluent, cells were passaged at a ratio of 1:3 to expand the cultures. The previous culture media was discarded from the flask and the cells were rinsed twice with pre-heated PBS. To detach the confluent cultured cells from the flask, trypsin/EDTA (0.05%/0.02%) was added for less than 1 min and the cells observed microscopically. The cells started rounding up i.e. losing cell-cell contacts. Then trypsin was removed. The flasks were then incubated at 37^0C for 1-2 min. Following incubation, the side of the flask was tapped against the palm in order to separate the cells from the surface of the flask. Detached cells were re-suspended in an appropriate volume of complete endothelial cell growth medium, cells then were seeded into the appropriate container T75 cm^2 flasks and returned to the incubator at 37^0C, 24hr later cells were washed with warm PBS and the medium was changed. All these steps were repeated once again when the cells reached confluence state.

2.2.2.4. Cryopreservation of cell stocks

In order to keep HUVEC from the same donor for future works, cells were cryopreserved between passage 2 and 4. After trypsinization as described above, cells were re-suspended in complete endothelial cell growth media

and centrifuged at 560g for 5 min at room temperature to sediment the cells. The cell pellet was re-suspended in 4^0C foetal bovine serum containing 10% v/v DMSO. Cells were frozen at a density of $1x10^6$ cells per ml. Cells were placed in cryovial and placed immediately on ice. The cryovials were wrapped in layers of tissue paper and placed into -80^0C for 24hr, and then the cryovials were transferred to liquid nitrogen Dewar flasks and stored until required.

2.2.2.5. Thawing and maintenance of HUVEC

The cryopreserved vial of HUVEC was removed from the liquid nitrogen storage tank and the cells thawed by placing the lower half of the vial in a 37^0C water bath for approximately 1 min. The vial was removed from the water bath and wiped dry. The cell suspension of 1 ml was removed from the vial into the T75 cm^2 flask which contained 10 ml of endothelial cell growth medium, and was incubated overnight to allow cell adhesion.

After 24hr, the culture media was discarded and the flask rinsed two times with 5 ml of pre-heated PBS, added to the side of the flask opposite the cells to avoid dislodging the cells and to remove any remaining media or detached cells. After this step, complete media was then added to the flask which was then returned to the cell incubator for further 48hr to allow the cells to become confluent.

2.2.2.6. Determination of cell concentration and cell viability

When required, the concentration of cells in suspension was determined by counting in a haemocytometer under an inverted phase contrast microscope. Cells were trypsinised off the T75 cm^2 flask, as described above and the

trypsinization was halted by adding 5-10 ml of complete media. Cell suspension was centrifuged at 560g for 5 min, after centrifugation the cell pellet was re-suspended in 5 ml fresh culture media. 10 µl of the cell suspension was removed from the 5 ml universal tube and was loaded into each chamber of a standard haemocytometer in order to allow the cells resolve on the grid, by using the inverted microscope as mentioned above. Cells were counted within four corners of the grid and the average was found. The cell concentrations were determined by using this formula:

Cell concentration (cells/ml) = total cell number (TC) / 4XDilution factor X 10^4

Total cells in the flask could then be determined by multiplying the total volume added to the culture media tube.

Trypan blue, a vital dye, was used to detect cell viability in HUVEC suspension. Viable cells exclude the trypan blue whilst this dye enters to the non-viable cells and which are easily identified during counting in the haemocytometer. Dyed cells and non-dyed cells were counted and the percentage viability was calculated as follows:

% viability = Unstained cells (viable)/total cells (viable and non-viable) x 100

2.2.2.7. Treatment of HUVEC with IV iron preparations

To investigate clinically relevant effects of IV iron preparation and to determine the effective concentration on HUVEC in culture, a dose response study was performed. HUVEC (2×10^5 cells per well) were cultured in complete endothelial cell growth medium and treated with three different concentrations of IV iron sucrose or IV FCM, which are known to be in the

clinically relevant range (50, 100, and 200 µg/ml) (Gupta *et al*., 2010; Kamanna *et al*., 2012; Martin-Malo *et al*., 2012) for 24hr at 37^0C in 5% CO_2. These concentrations were chosen and calculated based on the anticipated plasma concentrations that are achieved in CKD patients after 100 to 1000 mg IV iron sucrose injection which is close to 600 to 6000 µmol/l or 33 to 330 µg/ml plasma respectively and plasma concentrations close to 672 to 6720 µmol/l or 37 to 370 µg/ml respectively in patients injected with 100 to 1000 mg IV FCM (Van Wyck *et al*., 2004; Kamanna *et al*., 2012; Praschberger *et al*., 2013). After 24hr incubation, the number of viable cells was determined using trypan blue exclusion assay.

2.2.2.8. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay for HUVECs proliferation

To determine the HUVEC growth rate, an MTT assay was conducted. A solution of MTT is yellowish was widely used to measure cell viability and proliferation (Mosmann, 1983). Mitochondrial dehydrogenase of viable cells cleaves the Tetrazolium ring of MTT, yielding purple MTT formazan crystals. These crystals can be dissolved in DMSO and the resulting colour read spectrophotometrically giving absorbance proportional to cell number. However, MTT assay have HUVEC from passage 2-4 were used in this assay. Confluent HUVEC were trypsinised as mentioned above and were seeded in triplicate into gelatine-coated 96 well plates $1x10^4$ cells per well. Endothelial cell growth medium was added to three wells of the plate to act as a cell blank control. Cells were incubated overnight at 37^0C and 5% CO_2 in a humidified atmosphere to allow the cells attach to the surface of the plate. After 24hr, culture medium was changed and replaced with medium

containing 50 µg/ml IV iron sucrose or IV FCM. Plates were incubated at 37^0C and 5% CO_2 in a humidified atmosphere for 96hr and media was changed once after 48hr. Following incubation, MTT solution (5mg/ml) was added aseptically in an amount equal to 10% of the culture volume and plates were incubated for 3 to 4hr at 37^0C and 5% CO_2 in a humidified atmosphere. Subsequently, the media was discarded and the crystals formed were dissolved in 150 µl DMSO. The resulting purple colour was read within 1hr using Microplate reader MRX II (Dynex Technologies, Chantilly, USA) at a wavelength of 570nm and blank readings were subtracted from those samples, giving a proliferation curve over 96hr. This technique was also carried out every 24hr for 4 days to measure the daily proliferation rate of non-treated and IV iron preparations-treated HUVEC.

2.3. Statistical analysis

Excel and SPSS software 21.0 packages were used for the statistical analysis. For normally distributed values, all data analysis was carried out using paired *t*-test unless specified. Differences were examined for statistical significance using the one-way analysis of variance (ANOVA). The same statistical analysis was carried out in this chapter and all the following chapters unless specified. The differences were considered significant if p values were (< 0.05). Significant differences are marked with (*) $p< 0.05$, (**) $p< 0.01$ and (***) $p< 0.001$.

2.4. RESULTS

2.4.1. Culture of primary HUVEC

Under phase contrast microscopy, newly cultured HUVECs looked like small clumps of round cells, they adhered within 12hr to the gelatin coated flasks and under phase contrast microscope, they had a small clusters of elongated cells with thin peripheral cytoplasm, centrally located nucleus and indistinct borders (Figure 2.1A). These cells proliferated and became confluent within 3-4 days in 6 well plates and 7-9 days in T75 cm^2 flasks depending on the growth rate (Figure 2.1B). The growth rate was variable between cells from different donors. Total contact inhibition was not observed. However, cells proliferated very slowly when they reached confluence and the number of floating dead cells was higher in confluent flasks then non-confluent ones.

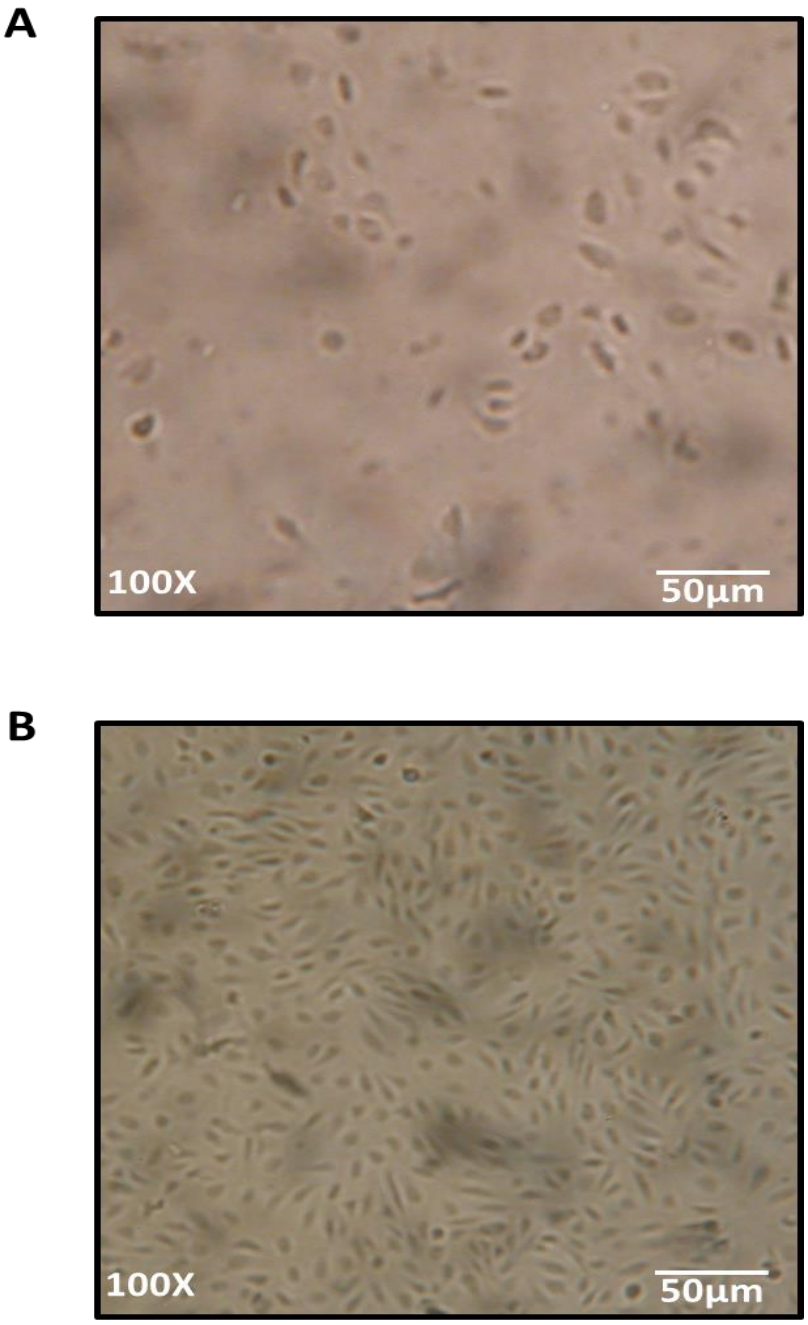


Figure 2.1: Morphology of HUVECs in culture. **A:** primary HUVEC after 24h of harvesting. **B:** confluent monolayer of HUVEC. (Original magnification is 100x).

2.4.2. Characterization of primary HUVEC

HUVEC phenotype was visualized using phase-contrast microscopy and immunostaining for CD31 (PECAM-1) in addition to the distinct cobblestone like arrangement of the cells at confluence as shown in (Figure 2.2). A FITC-tagged antibody against CD31 was used to visualise their expression on the HUVEC surface. Expression of CD31 molecule presented high fluorescence on surface of HUVEC, representing a basal expression of this molecule as shown in (Figure 2.2b). In contrast, HUVEC which were incubated with only FITC secondary mouse antibody (negative control) showed no fluorescence as shown in (Figure 2.2 (2b)).

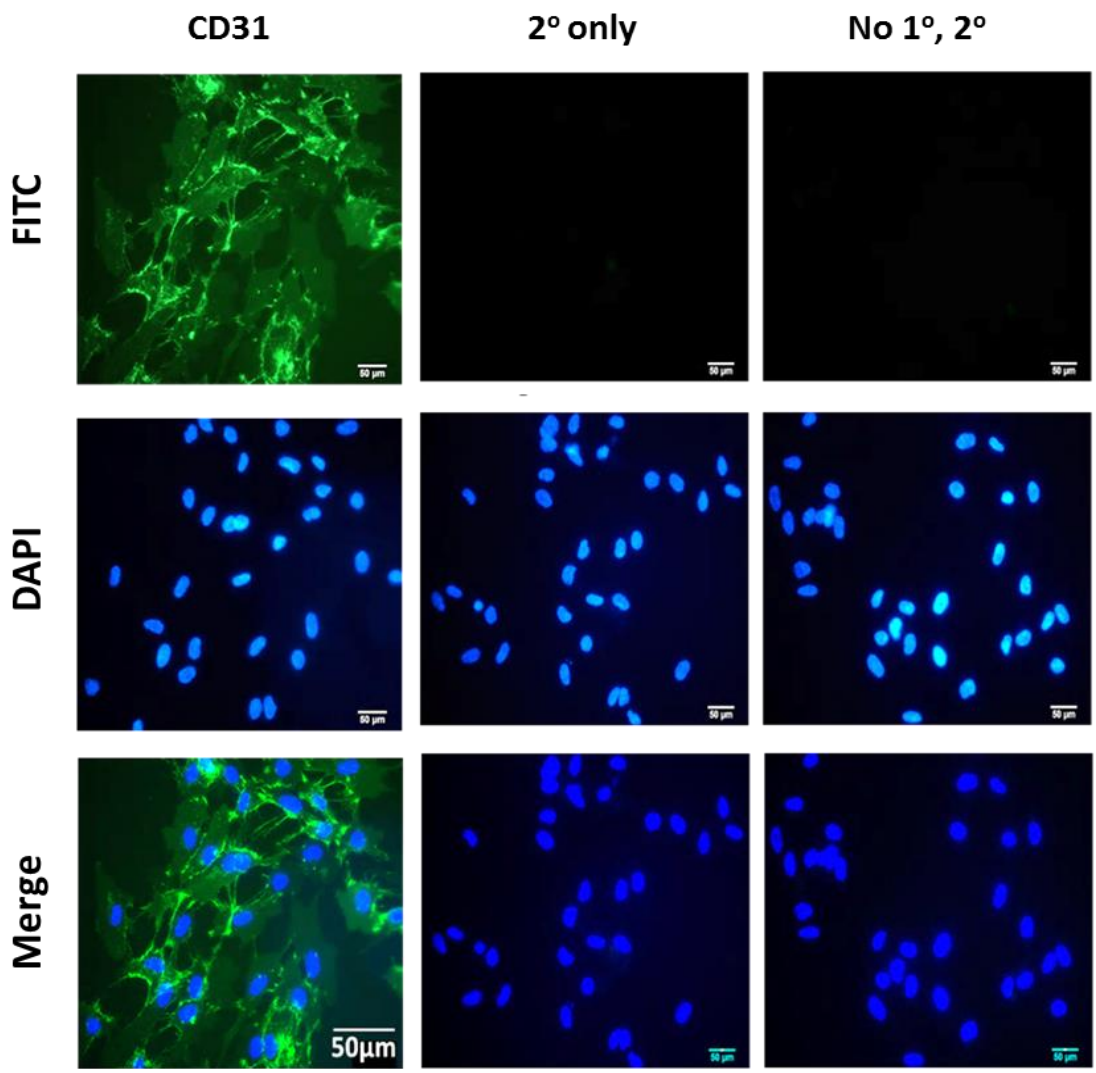


Figure 2.2: Representative images of the expression of CD31 on HUVEC surface. HUVEC were cultured in complete endothelial growth medium and then were fixed and immunostained with FITC-tagged antibodies against CD31. Clear specific detection seen for CD31. The upper row is immunofluorescence microscopy detection of DAPI. The middle row is immunofluorescence microscopy detection of CD31-FITC. The bottom one is a merge of the two. (Original magnification: 400X).

2.4.3. Treatment with IV iron preparations

Following 24hr of incubation with 50, 100 and 200 µg/ml of IV iron sucrose or IV FCM, whilst IV FCM demonstrated negligible toxicity at all concentrations, the IV iron sucrose was toxic to the HUVEC at both 100 and 200 µg/ml as shown in Figure 2.3. Some reduced viability was also seen at 50 µg/ml. Hence, 50 µg/ml was used in subsequent assays for both compounds.

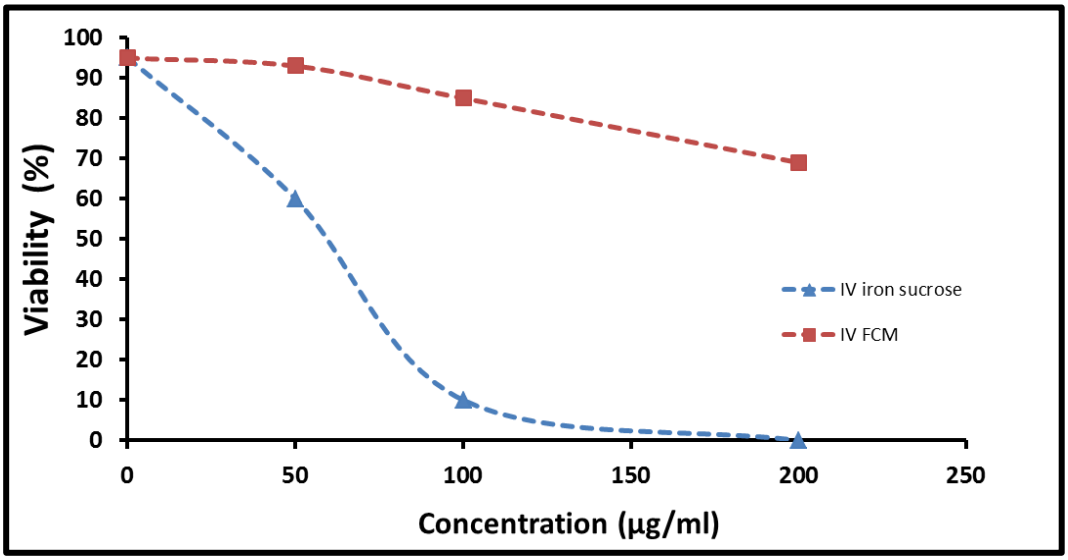


Figure 2.3: Dose response of IV iron preparations on cell viability. HUVECs were cultured in endothelial cell growth media in 6 well plates until cells attain about 80% confluent. Cells were treated with 50, 100 and 200 µg/ml IV iron sucrose for 24hr. Cellular viability was measured by trypan blue under phase contrast microscopy. The results are expressed as mean (+/- SEM) from 3 different donors (n=3).

2.4.4. Morphological changes in HUVEC cultured in IV iron preparations

To investigate the effect of IV iron preparations on HUVEC, we first observed the cell morphology of the HUVEC after exposure to two different types of IV iron preparations. Non-treated HUVEC showed normal morphology with typical cobblestone appearance as shown in Figure 2.4A. Following 24hr of incubation with 50 µg/ml of IV iron sucrose or IV FCM, the morphological changes in the HUVEC were observed as indicated in Figure 2.4C and 2.4B respectively. Obvious morphological changes were observed in the HUVEC compared with non-treated cells, the majority of the cells in the IV iron sucrose-treated HUVEC became shrunk and star-shaped with sharp outlines. These results suggest that IV iron sucrose causes cell rearrangement in HUVEC.

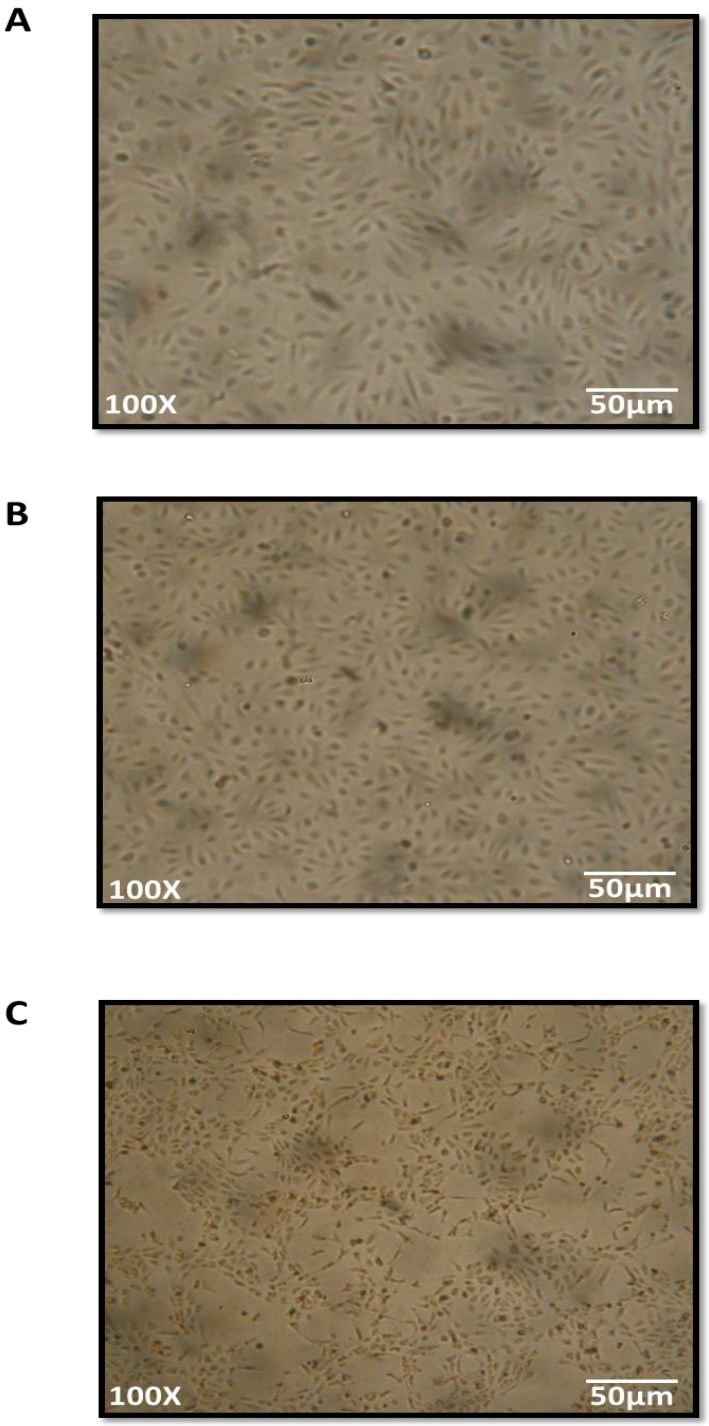


Figure 2.4: Morphological changes in HUVEC cultured with two different types of IV iron preparations (sucrose or FCM). **A:** cells were grown in 6 well plates in complete endothelial growth medium and considered as a control. **B:** HUVECs were incubated with 50 µg/ml of IV FCM for 24hr. **C:** HUVECs incubated with 50 µg/ml of IV iron sucrose for 24hr. (Original magnification: 100X).

2.4.5. Effects of IV iron preparations on HUVEC viability

There have been previously published reports that human arterial ECs (HAECs) treated with 10-100 µg/ml IV iron sucrose 4-24hr, causes decreased cell viability (Kamanna *et al.*, 2012). However, to our knowledge there have not been any *in vitro* published studies that investigate the effect of IV iron sucrose or IV FCM on HUVEC viability. Therefore, this test was performed to directly determine the effect of the two commercially IV parental iron preparations, IV iron sucrose or IV FCM on cellular viability of HUVEC under our laboratory conditions. HUVEC from the same donor were either treated with 50 µg/ml IV iron sucrose or IV FCM for 24hr or left untreated and considered as control. Trypan blue dye exclusion was used to distinguish between live and dead cells, and the percentage of live cells was more than 95% in all non-treated cells (Figure 2.5). However, percentage of live cells treated with 50 µg/ml of IV FCM or IV iron sucrose was 92% and 61% respectively after 24hr (Figure 2.5). Indicating that IV iron sucrose had toxic effects on HUVEC leading to reduced cell viability.

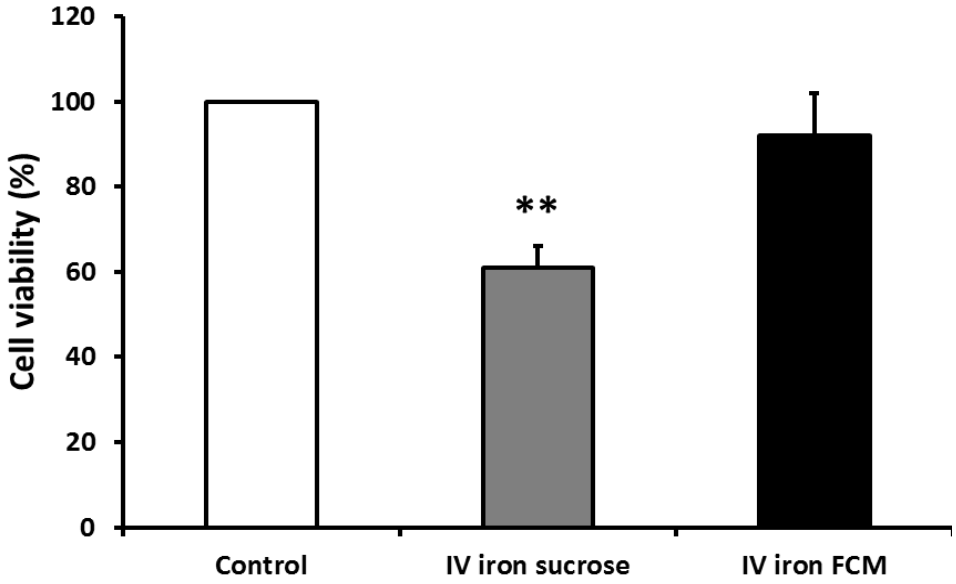


Figure 2.5: Effect of IV iron sucrose or IV FCM on HUVEC viability. Trypan blue dye exclusion test to show residual cell viability, HUVECs were cultured in endothelial cell growth media in 6 well plates for 2 days until cells attain about 80% confluent. Cells were treated with 50 µg/ml IV iron sucrose or IV FCM for 24hr or left untreated and considered as control. Cellular viability was measured by trypan blue under phase contrast microscopy. The results are expressed as mean (+/- SEM) from 3 different donors (n=3). ** $p<0.01$ compared with non-treated cells.

2.4.6. Characterization of IV iron preparation effects on HUVEC proliferation

The effects of IV iron preparations on HUVEC proliferation were evaluated by assessing mitochondrial dehydrogenase activity, using the MTT assay. Cell proliferation was assessed by monitoring the conversion of MTT to formazan (Figure 2.6). The reduction of MTT is catalysed by mitochondrial dehydrogenase enzymes and is therefore a measure for cell proliferation (Mosmann, 1983). HUVEC were seeded in 96 well plates at a concentration of $1x10^4$ cells per well in triplicate and incubated at 37^0C in 5% CO_2 for 96hr. The MTT assay was then conducted and the relationship between cell number and absorbance was determined (Figure 2.7). In addition, the MTT assay was repeated at 24hr intervals up to 96hr. There was a significant difference in the proliferation rate between non-treated cells and IV iron sucrose-treated cells (p<0.01 and p<0.001) at 48, 72 and 96hr respectively, while no significant difference was observed between IV iron sucrose-treated cells and non-treated cells at 24hr (Figure 2.8). HUVEC incubated with IV FCM at both 24 and 48hr showed no significant reduction in the growth rate compared with non-treated cells (Figure 2.8). In contrast, the growth rate of cells treated with IV FCM at 72 and 96hr did increase but was significantly lower than non-treated cells (p<0.05) (Figure 2.8).

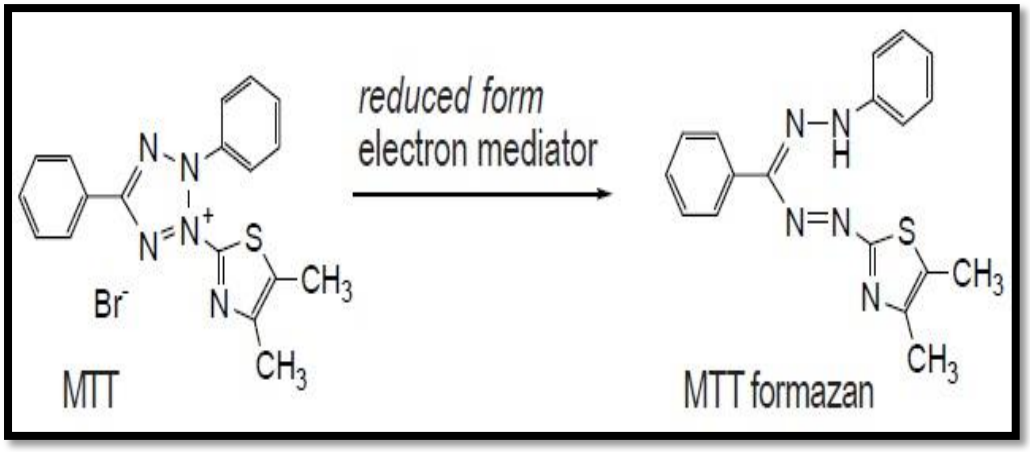


Figure 2.6: Representative image showing the reduction of yellow MTT to purple MTT formazan, was taken from Mosmann, 1983.

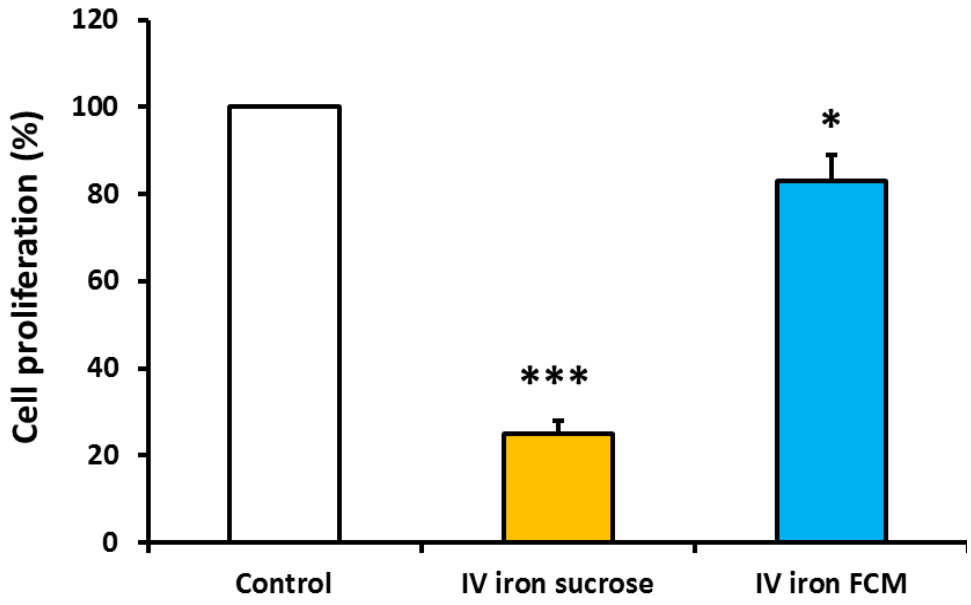


Figure 2.7: The effects of IV iron preparations on cell proliferation rate. HUVEC were treated with 50 µg/ml of IV iron sucrose or IV FCM. HUVEC were seeded in 96-well plates at density $1x10^4$ cells per well in triplicate and MTT assays were conducted after 96hr. Non-treated HUVECs from the same donor were used in each experiment as negative controls. The results are expressed as mean (+/- SEM) from 3 different donors (n=3). Non-treated absorbance values were defined as 100% and other values were adjusted accordingly. *p<0.05 and ***p<0.001 versus non-treated cells respectively.

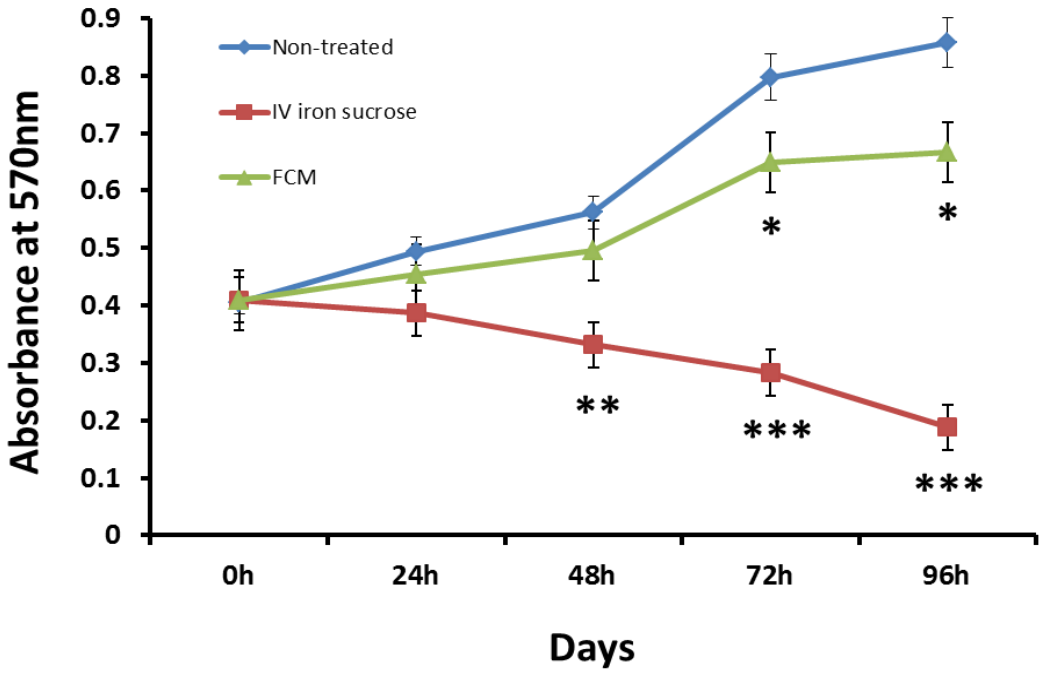


Figure 2.8: The effects of IV iron sucrose or IV FCM on HUVEC proliferation rate over 4 days. Cells were seeded in triplicate in 96 well plates at density $1x10^4$ cells per well. HUVECs were incubated for 96hr in the absence non-treated (control) or presence of 50 µg/ml IV iron sucrose or IV FCM. MTT assay was performed every 24hr for four days. Data are expressed as (mean+/- SEM) from 3 different donors (n=3). *p<0.05, **p<0.01 and ***p<0.001 compared with non-treated cells.

2.5. DISCUSSION

The results in this chapter showed that, IV iron sucrose induced morphological, functional changes include induced marked decrease proliferation and inhibited the survival of the HUVEC when compared with non-treated cells. In contrast, exposure of HUVEC to IV FCM did not promote EC dysfunction compared with non-treated cells.

Due to the limited availability of human vascular endothelial tissue and HAEC (Romanoski *et al*., 2010; Mäkinen *et al*., 2014), *in vitro* studies have used EC from a variety of sources to study the mechanisms of EC dysfunction that lie behind atherosclerotic plaque formation (Zhang *et al*., 2001a; Lee *et al*., 2002; Dalal *et al*., 2003; Ferretti *et al*., 2004). HUVEC represent one easily accessible *in vitro* model mainly because of umbilical cords availability and have played a major role as a model system for the study of the regulation of EC function which have been used successfully in a large number of studies (Outinen *et al*., 1999; Xu *et al*., 2000; Dong *et al*., 2005). HUVECs also represent a more relevant model than murine or bovine arterial EC which have been used by some groups (Weiss *et al*., 2002; Jin *et al*., 2007). This is because the umbilical vein carries oxygenated blood which delivers oxygen and nutrition to the placenta and HUVEC exposed to the same growth factors, cytokines and other factors to which arterial EC are exposed. Therefore, HUVEC physiology closely approaches that of arterial cells (Kivetys and Granger, 1997). In addition, the role of venous EC in acute inflammation is well established, as veins are the major site of leukocyte recruitment. In CKD there is systemic inflammation (Machowska *et al*., 2015) and therefore all types of ECs are likely to be activated. All these make the

use of HUVEC in these studies a convenient approach for investigating and determining the mechanism for IV iron preparation-induced EC damage important in CKD.

Characterization of HUVEC used in this study showed that these cells retain important endothelial characteristics until at least passage number four as defined by their basal expression of CD31 (PECAM-1) on their surface (Figure 2.2), and is in agreement with the findings of other researchers who showed that, these cells retain important endothelial characteristics in culture (Varani *et al*., 1995; Kaushal *et al*., 2001). Due to the possibility that, HUVEC may lose characteristic morphologic features, characteristics of apoptosis and DNA fragmentation, once passaged in culture for a longer time, cells were not used beyond this number of passage in culture in all studies for this thesis. Iron is important for the proliferation and survival of all cells and required to catalyse various redox reactions (Ganz, 2005). IV iron supplements are widely used to maintain adequate iron stores and manage to reduce the anaemia in CKD patients (Hörl, 2007). However, these compounds including IV iron sucrose has been shown inhibit proliferation and to cause EC injury, otherwise known as EC dysfunction, representing a key early step in the development of atherosclerosis (Agarwal *et al*., 2004; Kamanna *et al*., 2012; Masuda *et al*., 2013). Atherosclerosis is defined as a chronic inflammatory response in the walls of arteries (Lusis, 2000; Galkina and Ley, 2009), patients with CKD have higher risk of developing CVD, which is associated with atherosclerosis and is the major cause of death in patients treated with CKD (Ardhanari *et al*., 2014).

The initial aim of this study was to confirm the hypothesis that IV iron sucrose produces alterations on HUVEC morphology, viability, and growth as has been previously shown in other *in vitro* EC culture models and compare to treatment with the same clinically relevant concentrations of the more recently developed IV iron preparation, FCM. Figure 2.3A showed normal morphology of non-treated HUVEC with typical cobblestone appearance. However, HUVEC treated with 50 µg/ml IV iron sucrose for 24hr had lost their normal morphological characteristics, shrunk and were detached from the plates compared with non-treated cells (Figure 2. 4C). Data from trypan blue exclusion to determine cell viability and MTT assays confirmed these effects (Figures 2.4 and 2.7). In contrast, IV FCM-treated cells showed normal morphological characteristics compared with non-treated cells (Figure 2. 4B), and less severe effects on cell proliferation with little reduction in cell viability suggesting that HUVEC are more sensitive to damage by IV iron sucrose than IV FCM. This is consistent with a published study by Kamanna *et al* (2012) which showed that, treatment of HAEC with 50 and 100 µg/ml IV iron sucrose for 4hr had detrimental effects on cell morphology as the cells shrunk and detached from the plates, and the monolayer was disrupted. In addition, many cells exhibited typical features of apoptosis including condensed and fragmented nuclei when compared to non-treated cells. These authors suggested that, alteration of ECs morphology could be due to the increased in plasma level of catalytically active NTBI or free iron after IV iron being administered. This NTBI in turn is capable of generating oxidative stress, which causes endothelial injury and dysfunction, and triggers inflammation via activation of NF-κB. Interestingly, from Kamanna study it

seems that HAEC were more sensitive to IV iron sucrose than our model of HUVEC. The authors showed that the morphology was altered at 4hr of treatment with 50 µg/ml iron sucrose, and the cells exhibited typical features of apoptosis such as, condensed or fragmented nuclei as compared with non-treated cells. However, our data is similar to earlier published work by Kamanna and others, that treatment with IV iron sucrose caused shrunk and star-shaped with sharp outlines in HUVEC morphology (Figure 2. 4C). In addition, IV iron sucrose significantly reduced the viability of HUVEC by almost 40% compared with non-treated cells at 24hr (p<0.01) (Figure 2.5), which indicate that IV iron sucrose was toxic to HUVEC. This is another evidence shows that HAEC is more sensitive to IV iron sucrose compared to our data of HUVEC, as 61% of HUVEC were viable after treatment with IV iron sucrose for 24hr. A study by Masuda *et al* (2014) showed that, treatment of isolated pancreatic islet cells with 20, 100 and 200 mg/l IV iron sucrose resulted in significant decrease in cell viability using tetramethylrhodamine ethyl ester (TMRE) methods. These authors reported that the reduction in islet cell viability occurs as a result of increased oxidative stress. Kamanna *et al* (2012) also showed that treating HAEC with 10, 50 and 100 µg/ml IV iron sucrose for 24hr, significantly decreased cellular viability using MTT assay compared with non-treated cells with viability being reduced to 20% on treatment with 50 and 100 µg/ml for 4hr. In contrast, cells treated with 50 µg/ml of IV FCM showed that this component was not cytotoxic to cells since the viability of cells was always greater than 92% with the number of dead cells similar to that observed in non-treated (Figure 2.4). Thus, not all ECs have the same response to IV iron compounds and this may explain the

differences in the results between diverse studies from the literature. These differences in cell survival over 24hr may result from pharmacokinetic differences such as molecular weight, reactivity, thermodynamic stability and half-lives between IV iron sucrose or IV FCM, which differ in their carbohydrate ligands as discussed in section 1.6.3.1. These differences result in a substance-specific kinetic of NTBI release from iron complexes (Fell *et al*., 2014).

Fell *et al.,* (2014) examined the effect of 100, 200 and 500 µg/ml IV iron sucrose or IV FCM for 3hr, on monocyte cell viability by Annexin V binding buffer using flow cytometry method. The authors found a significant reduction in cell viability with IV iron sucrose treatment versus non-treated cells, which was not the case in cell treated with IV FCM, as there was no significant decrease in the cell viability compared with non-treated cells. Fell *and* others suggested that the reduction of cell viability by IV iron sucrose was due to the differences between IV iron sucrose and IV FCM in term of properties such as carbohydrate ligands, molecular weight and half-lives. IV iron sucrose has the lowest molecular weight, the shortest half-life and the lowest stability compared with IV FCM, which lead to the release of higher levels free iron or NTBI (Jahn *et al*., 2011; Geisser and Burckhardt, 2011; Nordfjeld *et al*., 2012). This in turn may induce distinct toxic effects such as ROS production and subsequently DNA damage (Fell *et al.,* 2014).

There are numerous methods available to study cell proliferation including MTT assay and [3H] thymidine incorporation. However, [3H] thymidine incorporation has many limitations compared to MTT assay including dependence upon cellular uptake, extra nuclear binding of thymidine,

problems with the physiochemical properties of the radioactive [3H] thymidine as reviewed by Maurer (1981). Thus, in order to confirm our finding by trypan blue method, the proliferation of HUVEC treated with IV iron preparations was investigated using MTT assay. The significant increased reduction of MTT by treated cells provide a sign of the disrupted mitochondria function, which in turn can lead to apoptosis of ECs. Although we know that MTT results depend on mitochondrial function and oxidative stress can compromise that, the data in our lab by Sultan and others showed hyperglycaemia induced oxidative stress but effects on proliferation were similar when measured by MTT or manual counting (Sultan *et al*., 2015).

Consistent with our findings, other studies showed that IV iron sucrose reduced EC proliferation using either [3H] thymidine incorporation or MTT methods. Zager *et al* (2002) showed that IV iron sucrose inhibits HAEC proliferation when these cells were treated with 60 and 1000 µg/ml for 72hr using MTT assay. In addition, another study was done by Carlini *et al* (2006), who agreed with the finding of Zager *et al* and Carlini *and* others, treating bovine aortic ECs (BAECs) with 500 and 1000 µg/ml IV iron sucrose for 24 and 48hr showed that this complex significantly decreased BAEC proliferation compared with non-treated cells using [3H] thymidine incorporation assay. These groups suggested that impaired mitochondria function was the cause for inhibition of HAEC proliferation by this compound.

All the previous studies reflect the complex biochemical events initiated by the iron preparation on the cells, which could include the loss of mitochondria integrity and the production of oxidative stress. This needs further investigation which will be carried out in the next chapters to confirm and

quantify the generation of oxidative stress by IV iron sucrose or IV FCM. It has been shown that the majority of patients with CKD die due to CVD.

The key early role of EC dysfunction in generating atherosclerosis is a contributing factor to CV disease. The findings of this study showed that both IV iron compounds have the capacity to reduce HUVEC proliferation and this could be contributing factor to CVD leading to CKD death but the extent and mechanisms responsible for the damage require further investigation.

2.6. CONCLUSION

IV FCM has been shown to be less harmful than IV iron sucrose at pharmacologically relevant concentrations and did not kill HUVEC in culture. However, our study is the first *in vitro* study to show that IV FCM still caused decreased EC proliferation rate especially when incubated with cells for longer time (72 and 96hr). In contrast, IV iron sucrose significantly induced changes in cell morphology and inhibited proliferation of cultured HUVEC compared with untreated cells. These factors need to be taken into account when administering iron preparations to patients with CKD. Based on these findings our study went to investigate mechanisms which may be responsible for altered HUVEC morphology and growth.

CHAPTER 3

THE ROLE OF OXIDATIVE STRESS IN IV IRON PREPARATION-MEDIATED EC DYSFUNCTION

3.1. INTRODUCTION

Oxidative stress is a cellular condition which results from high levels of ROS such as superoxide anion and/or either impaired or defective function of antioxidant defence, such as SOD and GPx (Sies, 1997; Schnabel and Blankenberg, 2007), and it has been shown in many studies to be associated with CVD (Cai and Harrison, 2000; Schnabel and Blankenberg, 2007). Under physiological conditions, ROS production is tightly regulated to participate in the regulation of cellular activities and functions such as cell proliferation, differentiation and cell signalling (Kamata and Hirata, 1999; Trachootham *et al*., 2008) as described in chapter 1 (section 1.2.3). However, overproduction of ROS can generate oxidative stress, which leads to cellular damage and CV remodelling (Hamza and Dyck, 2014).

Because anaemia is a significant source of morbidity and mortality in ESRD patients (Kausz *et al*., 2000), iron deficiency is an important clinical concern which is prevalent in patients with CKD and contributes to anaemia (Eschbach *et al*., 1977). Iron can exert potent pro-oxidant effects due to its ability to donate or accept an electron (Flora, 2009). In the Fenton reaction, H_2O_2 a relatively innocuous and easily containable ROS, is converted by iron to the hydroxyl radical, which is the most reactive and cytotoxic ROS (Kamanna *et al*., 2012). Therefore IV iron preparation administration can induce the formation of ROS in CKD patients and cause oxidative stress producing inflammation, endothelial injury and dysfunction (Duffy *et al*., 2001; Pai *et al*., 2011; Brissot *et al*., 2012; Kamanna *et al*., 2012), which may be partially mediated by LDL oxidation and antioxidant depletion (Valenti *et al*., 2007). Oxidative stress in patients with CKD can cause progression of renal

disease and cardiovascular morbidity and mortality (Himmelfarb *et al*., 2002; Agarwal *et al*., 2004). Several *in vivo* (Zager *et al*., 2002; Agarwal *et al*., 2004; Bishu and Agarwal, 2006), *in vitro* (Shah, 2001) and clinical studies (Lim *et al*., 1999; Herrera *et al*., 2001; Showkat *et al*., 2014), have demonstrated that IV iron preparations increase oxidative stress biomarkers such as MDA*,* AOPP, SOD*,* glutathione peroxidase and lipid hydroperoxide (LHP). In addition, oxidative stress was shown to be increased after IV iron sucrose was administered in CKD patients at stage 3 and 4, which resulted in increase in proteinuria and tubular injury (Agarwal *et al*., 2004). It has been found that, within 15 to 30 min after 100 mg of IV iron sucrose administration to CKD patients, a rapid increase of MDA resulted (Showkat *et al*., 2014). Furthermore, in patients with CKD, markers of oxidative stress such as LOOH and diene conjugates (DC) have been found to correlate with EC dysfunction, an early critical step in the pathogenesis of atherosclerosis and CVD (Annuk *et al*., 2001).

Additionally, IV iron preparation can cause renal injury due to direct drug-induced toxicity including changes in glomerular hemodynamics, tubular cell toxicity, inflammation, crystal nephropathy, rhabdomyolysis, and thrombotic microangiopathy (Kim and Moon, 2012). This injury induced by IV iron preparation *per se* can cause the production of oxidative stress (Bhandari, 2011). IV iron can cause oxidative stress induction in a number of cell types for instance; an *in vitro* study by Sturm *et al* showed that IV iron sucrose, ferric gluconate and iron dextran can all increase ROS production in HepG2 cells (Sturm *et al*., 2010). Zager *et al* have shown *in vitro* that, IV iron

preparations can induce production of ROS in cultured human proximal tubular cells and proximal tubular segments (Zager *et al*., 2004).

Increased ROS mediated endothelial injury can be possibly improved using an antioxidant treatment such as NAC (Lim *et al*., 1999; Agarwal *et al*., 2004). NAC has been found to reduce EC dysfunction by the reduction of ROS-dependent activation of NF-kB which was caused by uraemic toxins (Tumur *et al*., 2010). Furthermore, NAC has been found to reduce levels of the kidney oxidative stress marker MDA in a mouse model of diabetic nephropathy (Ribeiro *et al*., 2011). To our knowledge ROS production *in vitro* in HUVEC treated with IV iron preparations (either iron sucrose or FCM) have not previously been investigated.

Therefore, the aim of this chapter is to investigate the relative abilities of IV iron sucrose or IV FCM to induce ROS production in both an endothelial cell line (EA.hy926 cell line) and primary HUVEC by measurement of oxidant stress using DCFH-DA staining by flow cytometry method. In addition, NBT assay method was used to quantify superoxide anion generation in primary HUVEC. Furthermore, the ability of antioxidant treatment to reduce levels of IV iron induced superoxide anion and total ROS in HUVEC will be examined.

3.2. MATERIALS AND METHODS

3.2.1. Materials

3.2.1.1. Reagents

Dulbecco's modified eagle's medium (D-6546) and *N*-acetyl cysteine (NAC) (A7250) were purchased from Sigma-Aldrich Chemical Company (Poole Dorset, UK). 2′, 7′-Dichlorofluorescin Diacetate (DCHF-DA) (287810) and *p*-Nitroblue Tetrazolium Chloride (NBT) (484235) were supplied by Calbiochem (La Jolla, USA). Phenol red-free M199 media (11043-023) was supplied by Invitrogen (Paisley, UK). Dulbecco's Phosphate Buffer Saline (DPBS) (SH 3026401) was purchased from Thermo Fisher Scientific (Hemel Hempstead, UK). Foetal Bovine Serum (FB-1090) was purchased from Biosera (East Sussex, UK). Sample Test Tubes for Flow Cytometers, 12 x 75 mm (2523749) were purchased from Beckman Coulter (High Wycombe, UK).

3.2.1.2. Cell culture

All tissue culture reagents used were of tissue culture grade. All tissue culture plastics used (T75), 96 multi-well plates and 6 well plates were obtained from (corning, incorporated, costar, USA). Disposal sterile pipettes of volume 5, 10 and 25 ml from (Sarstedt company, France).

EA.hy926 cell lines were kindly provided by Dr. Anne Graham (University of Bradford). All cell culture procedures were performed using aseptic techniques in biological safety cabinet. All culture were preserved in a humidified in 5 % CO_2, 95% humidified air and at 37^0C.

All cells culture materials of HUVECs were as described in chapter 2 in (section 2.2.1.1).

3.2.2. Methods

3.2.2.1. Endothelial cell culture

3.2.2.1.1. Culture of EA.hy926 cell line

EA.hy926 cell lines were first established by Edgell *and* colleagues and were generated by fusing primary HUVECs with the human lung carcinoma cell line (A549) (Edgell *et al*., 1983). EA.hy926 cells were a generous gift from Dr. Anne Graham (University of Bradford). EA.hy926 cells were maintained and grown in DMEM media (catalogue number D6546; Sigma Aldrich), propagated in plastic tissue culture flasks T75 cm^2 supplemented with 10% foetal bovine serum (v/v), penicillin/streptomycin (100U/100 mg/ml) and L-glutamine (2 mM), cultured at 37^0C in a humid atmosphere containing 5% CO_2 in air. After reaching confluence, the cells were sub-cultured and reseeded. After 24hr the medium was discarded, and cells were washed twice with warm PBS to remove any dead cells. The cultures were then successfully grown *in vitro* using conditions above. When confluence was achieved, cells were passaged (every 5-7 days).

3.2.2.1.2. Culture of primary HUVEC

HUVEC were cultured as previously described in chapter 2, section (2.2.2.1).

3.2.2.2. Measurement of oxidative stress in ECs

3.2.2.2.1. Total ROS detection in EA.hy926 by flow cytometry using DCFH-DA staining

EA.hy926 cells were seeded into 6 well plates in complete medium at a density of $2x10^5$ cells per well and cultured until they reached 70-90% confluence. Media was aspirated and cells were washed twice with PBS to remove any dead cells from the plate. Cells were then treated with 100 or 500 µg/ml IV iron sucrose or IV FCM for 2, 6 and 24hr. Subsequently, media was discarded and cells were washed once with DPBS. Media without phenol red containing 10 µM DCFH-DA was added and cells were incubated at 37^0C and 5% CO_2 for 30-45 min. Subsequently, Media aspirated and cells were washed once with DPBS. Cells were then harvested in trypsin/EDTA (0.05%/0.02%) at 37^0C and 5% CO_2 for 2-3 min, the cells then were re-suspended in 1 ml ice-cold PBS in flow cytometry test tube on ice in the dark.

DCFH-DA is commonly used to measure oxidative stress in cells due to the high sensitivity of fluorescence-based assays, is non-fluorescent lipid compound and membrane–permeable, once it gets into the cell; it is converted into non-fluorescent DCFH through the action of cellular esterases, which stay trapped within the cells (Tarpey and Fridovich, 2001). However, in the presence of ROS such as H_2O_2, superoxide anion, hydroxyl and NO, DCFH is speedily oxidized to its fluorescent derivative 2', 7'-dichlorofluorescin (DCF) (Royall and Ischiropoulos, 1993) as shown in (Figure 3.1). The fluorescence intensity of DCF was read at 535 nm emission when excited at 488nm by flow cytometry (Beckman Coulter, Inc. UK).

3.2.2.2.2. Total ROS detection in HUVEC by flow cytometry using DCFH-DA staining

HUVEC were seeded into gelatin coated 6 well plates in complete endothelial cell growth medium at a density of $2x10^5$ cells per well and cultured until they reached 80-90% confluence. Culture medium was aspirated and the cells were washed twice with PBS. HUVEC were then treated with 50 µg/ml IV iron sucrose or IV FCM for 2, 6 and 24hr. Subsequently, endothelial growth media was discarded and cells were washed once with DPBS. Media without phenol red containing 10 µM DCFH-DA was added and cells were incubated at 37^0C and 5% CO_2 for 30-45 min. Subsequently, Media aspirated and cells were washed once with PBS. HUVEC were then harvested in trypsin/EDTA (0.05%/0.02%) at 37^0C and 5% CO_2 for 2-3 min, the cells then were re-suspended in 1 ml ice-cold PBS in flow cytometry test tube on ice in the dark. All individual donor samples were prepared and analysed in triplicate.

3.2.2.2.3. Detection of intracellular superoxide anion production in HUVEC by *p*-Nitro Blue Tetrazolium (NBT) assay

The NBT reduction assay was used to demonstrate whether IV iron sucrose or IV FCM, induce the generation of superoxide anions intracellularly in HUVEC. NBT forms a water-insoluble blue formazan crystal when it reacts with intracellular superoxide anion demonstrating the degree of superoxide formation within the cell (Auclair and Voisin, 1985). The formed crystals are trapped within the cell, however, can be released by directly lysing the cells with lysis solution. The formation of blue formazan can be measured spectrophotometrically at 570 nm.

Briefly, HUVEC were plated into gelatin coated 24 well cell culture plates in complete endothelial cell growth medium at a density of $5x10^4$ cells per well and incubated at 37^0C and 5% CO_2, medium was changed every 48hr until cells were 80-90% confluent. The cells were washed twice with warm PBS to remove any dead cells from the plate then HUVEC were treated with 50 µg/ml IV iron sucrose or IV FCM for 2, 6 and 24hr. Media then was aspirated and replaced with phenol red-free media (M199) containing 1 mg/ml NBT with and without 50 µg/ml of IV iron sucrose or IV FCM, the cells then were incubated at 37^0C and 5% CO_2 for 90 min. Preparation of cell lysate is a critical step and, therefore, it was essential to optimise the protocol to get the right lysis buffer. Thus, the first protocol used was as follow: HUVEC were harvested in 1 ml PBS by gentle scraping using a sterile scraper. The cell pellet was collected by centrifuging at 13000x g for 5 min. Pellets were lysed vigorously in 500 µl 100% pyridine and then cell lysis vortexed thoroughly before heating in a heating block at 95°C for 20 min. Absorbance was measured using a spectrophotometer at a wavelength of 570nm. However, this protocol did not work and no results were obtained. Subsequently, another lysis buffer recommended by Eligini *et al* (2005) was used, which led to the final data of NBT assay presented. HUVEC were directly lysed with lysis solution (90% DMSO, 0.1% SDS and 0.01 M of NaOH) (Eligini *et al*., 2005). The resulting blue coloured solution was measured spectrophotometrically at a wavelength of 750nm using Microplate reader MRX II (Dynex Technologies, Chantilly, USA). The production of intracellular superoxide anion was proportional to the absorbance values at 750nm. All samples were prepared and analysed in triplicate.

3.2.2.3. Treatment with antioxidants

Previously published work has shown that, co-treatment with antioxidant such as, NAC can effectively reduce ROS production in cells and patients treated with IV iron sucrose (Lim *et al*., 1999; Agarwal *et al*., 2004).

In order to investigate the role of antioxidant treatment on oxidative stress generated by IV iron sucrose or IV FCM, obtained by NBT assay or DCFH-DA staining cells were treated with NAC in the presence of absence of IV iron preparations. For DCFH-DA staining by flow cytometry, HUVEC were cultured for 6 and 24hr in complete endothelial cell growth medium containing 50 µg/ml either IV iron sucrose or IV FCM alone or in the presence of 2 mM NAC. For NBT assay, HUVECs were cultured for 2, 6 and 24hr in complete endothelial cell growth medium containing 50 µg/ml either IV iron sucrose or IV FCM alone or in the presence of 2 mM NAC. HUVEC isolated from the same patient were cultured for 24hr in complete medium alone, and were considered as a control for both NBT assay and DCFH-DA staining method as described in sections 3.2.2.2.2 and 3.2.2.2.3.

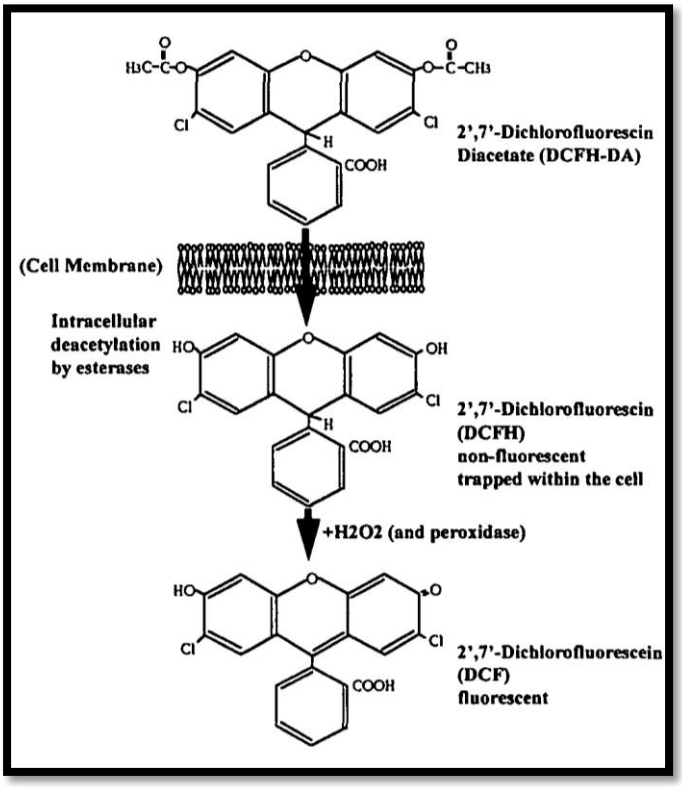


Figure 3.1: The mechanism of DCFH-DA staining, taken from Takanashi *et al*., 1997.

3.3. RESULTS

3.3.1. Detection of total intracellular ROS production in EA.hy926 using DCFH-DA staining

In order to investigate the role of IV iron sucrose or IV FCM on the generation of total ROS production in ECs, EA.hy926 cell line were initially used to optimize the methods of flow cytometry using DCFH-DA staining and determining dose response effects. Whether the EA.hy926 cell line can be a good model compared with primary HUVEC for studying the effect of these compounds on oxidative stress generation in cultured ECs was also a key consideration and is discussed later in the chapter. Figures 3.2B, 3.3B and 3.4B showed that non-treated cells at 2, 6 and 24hr showed very low levels of DCF fluorescence intensity. EA.hy926 cells treated with 100 µg/ml IV FCM or IV iron sucrose at 2hr showed a slight increase in ROS production compared with non-treated cells, however this increase was not significantly different compared with non-treated cells (Figure 3.2B). In contrast, cells treated with 500 µg/ml IV iron sucrose or IV FCM for 2hr showed significant increases in ROS production compared to non-treated cells ($p<0.05$) (Figure 3.2B). Figure 3.3B showed significant increase in ROS production in cells treated with 100 or 500 µg/ml IV iron sucrose or IV FCM at 6hr compared with non-treated cells ($p<0.05$) and ($p<0.01$) respectively. Furthermore, EA.hy926 cells which had been treated with 100 µg/ml IV iron sucrose or IV FCM, and those treated with 500 µg/ml IV FCM at 24hr showed significant increase in ROS production compared with non-treated cells ($p<0.01$) (Figure 3.4B).

Additionally, EA.hy926 cells treated with 500 µg/ml IV iron sucrose for 24hr showed a significant increase in ROS formation compared with non-treated cells (p<0.001) (Figure 3.4B). There was also a significant difference between those cells treated with 500 µg/ml IV iron sucrose and cells treated with IV FCM at 24hr (p<0.05) (Figure 3.4B). All these observations indicate that IV iron sucrose or IV FCM significantly induce oxidative stress generation in EA.hy926.

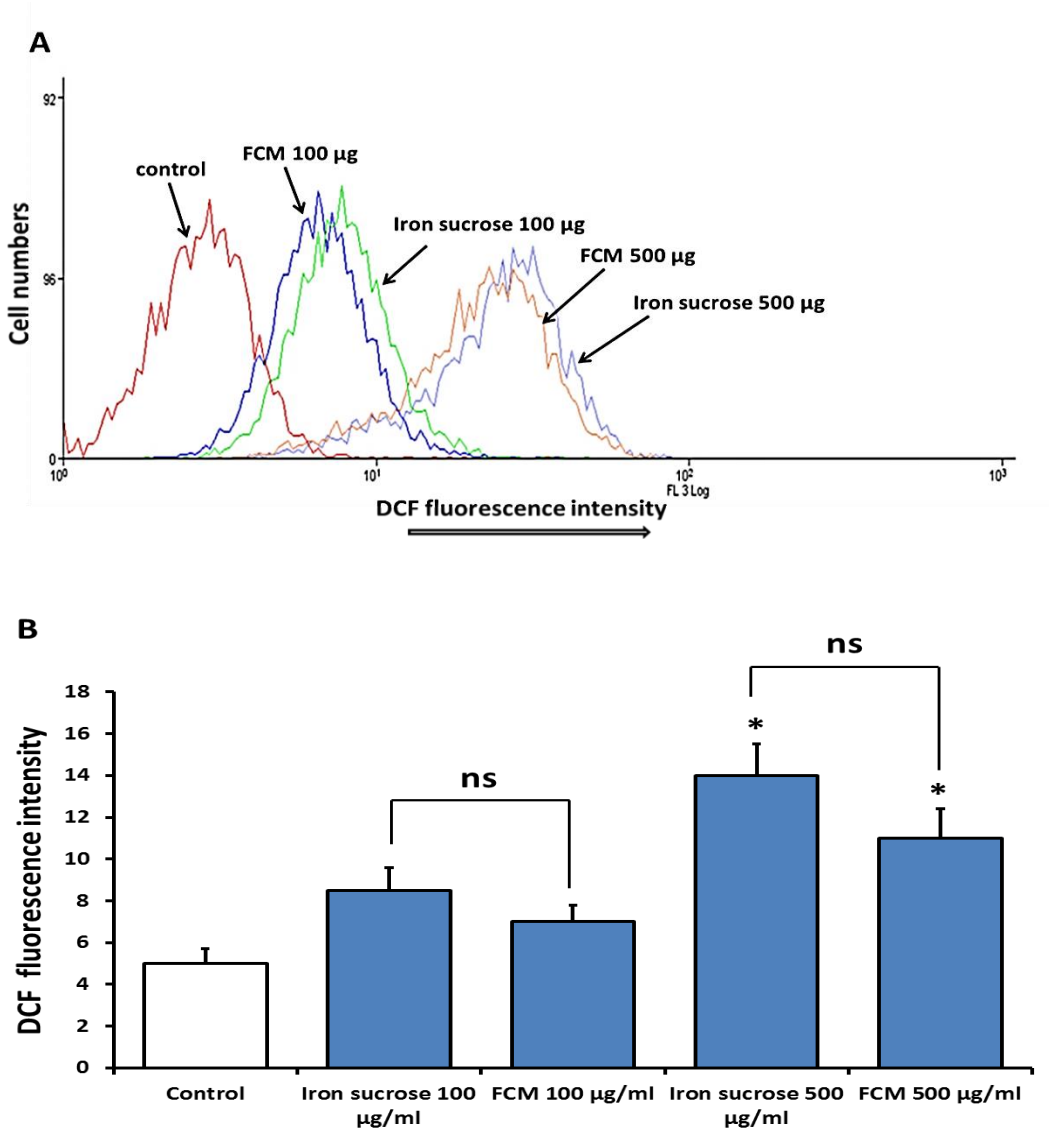


Figure 3.2: The effects of IV iron administrations on intracellular total ROS production in cultured EA.hy926 endothelial cell line at 2hr. EA.hy926 cells were washed with PBS and incubated with 10 µM DCFH-DA for 30-45 min. Then cells were either non-treated or treated with 100 or 500 µg/ml IV iron sucrose or IV FCM for 2hr. Non-treated cells were considered as negative control. **(A):** representative flow cytometry histogram of EA.hy926 cell line labelled with DCFH-DA staining. **(B):** DCF fluorescence intensity obtained from the histogram statistics. The results are expressed as mean (+/- SEM) from 3 different experiments (n=3). Fluorescence intensity of DCF was determined at 535nm emission when excited at 488nm by flow cytometry. *p<0.05 compared with non- treated cells.

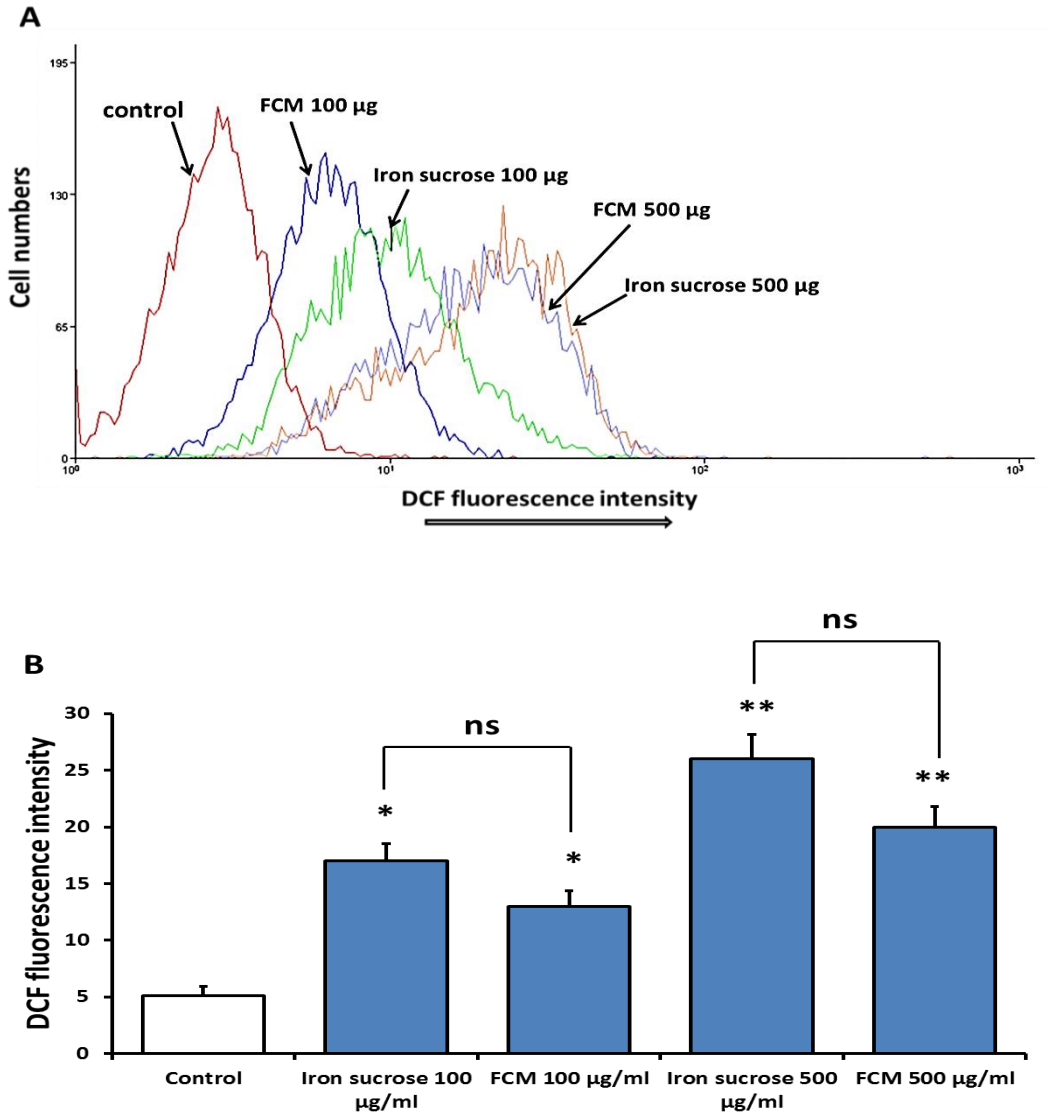


Figure 3.3: The effects of IV iron administrations on intracellular total ROS production in cultured EA.hy926 endothelial cell line at 6hr. EA.hy926 cells were either non-treated or treated with 100 or 500 µg/ml IV iron sucrose or IV FCM for 6hr. Then cells were washed with PBS and incubated with 10 µM DCFH-DA for 30-45 min. Non-treated cells were considered as negative control. **(A):** representative flow cytometry histogram of EA.hy926 cell line labelled with DCFH-DA staining. **(B):** DCF fluorescence intensity obtained from the histogram statistics. The results are expressed as mean (+/- SEM) from 3 different experiments (n=3). Fluorescence intensity of DCF was determined at 535nm emission when excited at 488nm by flow cytometry. **p*<0.05 and ***p*<0.01 compared with non-treated cells.

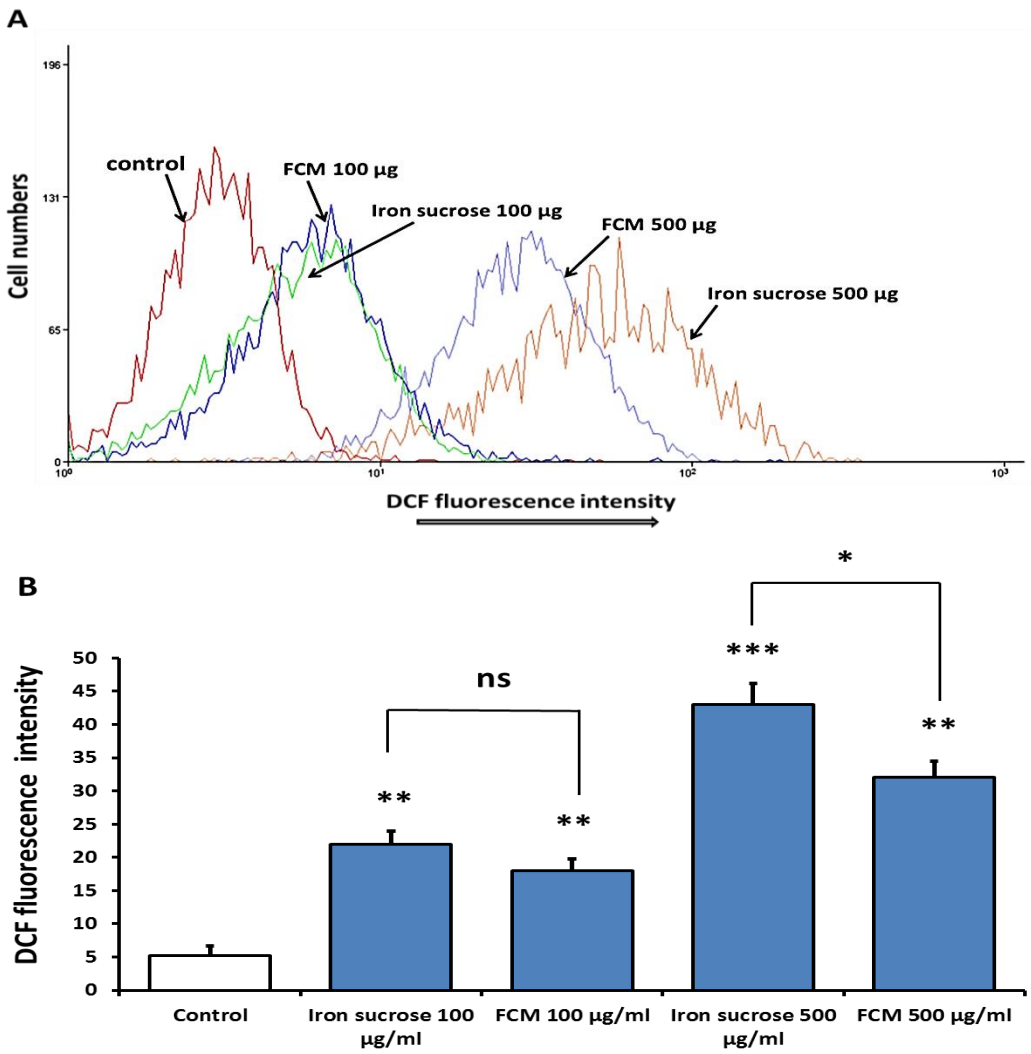


Figure 3.4: The effects of IV iron administrations on intracellular total ROS production in cultured EA.hy926 endothelial cell line at 24hr. EA.hy926 cells were either non-treated or treated with 100 or 500 µg/ml IV iron sucrose or IV FCM for 24hr. Then cells were washed with PBS and incubated with 10 µM DCFH-DA for 30-45 min. Non-treated cells were considered as negative control. **(A):** representative flow cytometry histogram of EA.hy926 cell line labelled with DCFH-DA staining. **(B):** DCF fluorescence intensity obtained from the histogram statistics. The results are expressed as mean (+/- SEM) from 3 different experiments (n=3). Fluorescence intensity of DCF was determined at 535nm emission when excited at 488nm by flow cytometry. ***p*<0.01 and ****p*<0.001 compared with non-treated cells. **p*<0.05 IV iron sucrose compared with IV FCM treated cells at 24hr.

3.3.2. Detection of total intracellular ROS production and the effect of NAC on ROS generation in primary HUVEC using DCFH-DA staining

In addition to superoxide anion, EC can produce different types of ROS such as H_2O_2 and peroxides (Dröge, 2002). In order to investigate whether IV iron sucrose or IV FCM might induce increases in the generation of total intracellular ROS production, total ROS level was determined in HUVEC by measuring the oxidative conversion of DCFH-DA to fluorescent compound DCF (Jia *et al*., 2006; Lin *et al*., 2002).

Figure 3.5B shows that non-treated cells produced small amounts of total ROS. IV iron sucrose or IV FCM at 2hr showed detectable increase in ROS level. However, this increase was not significant compared to non-treated cells (Figure 3.5B). IV iron sucrose or IV FCM-treated cells at 6hr showed a much higher production of the fluorescent dye versus to non-treated cells, which reflects significant increase in total ROS formation intracellularly (p<0.05). In addition, ROS production was significantly increased in HUVEC treated with IV iron sucrose or IV FCM at 24hr compared with non-treated cells (p<0.001) and (p<0.01) respectively (Figure 3.5B). HUVEC treated with IV iron sucrose or IV FCM and 2 mM NAC at 6 and 24hr showed significant reductions in ROS levels compared with the same cells treated with the IV iron sucrose or IV FCM alone at 6 and 24hr (p<0.05) (Figures 3.6B, 3.7B and 3.8B) and (p<0.01) (Figure 3.9B). However, although there was a reduction in ROS in IV iron sucrose or IV FCM-treated cells with NAC, this was still shown to be significantly higher when compared with non-treated cells (p<0.05) (Figures 3.8B and 3.9B).

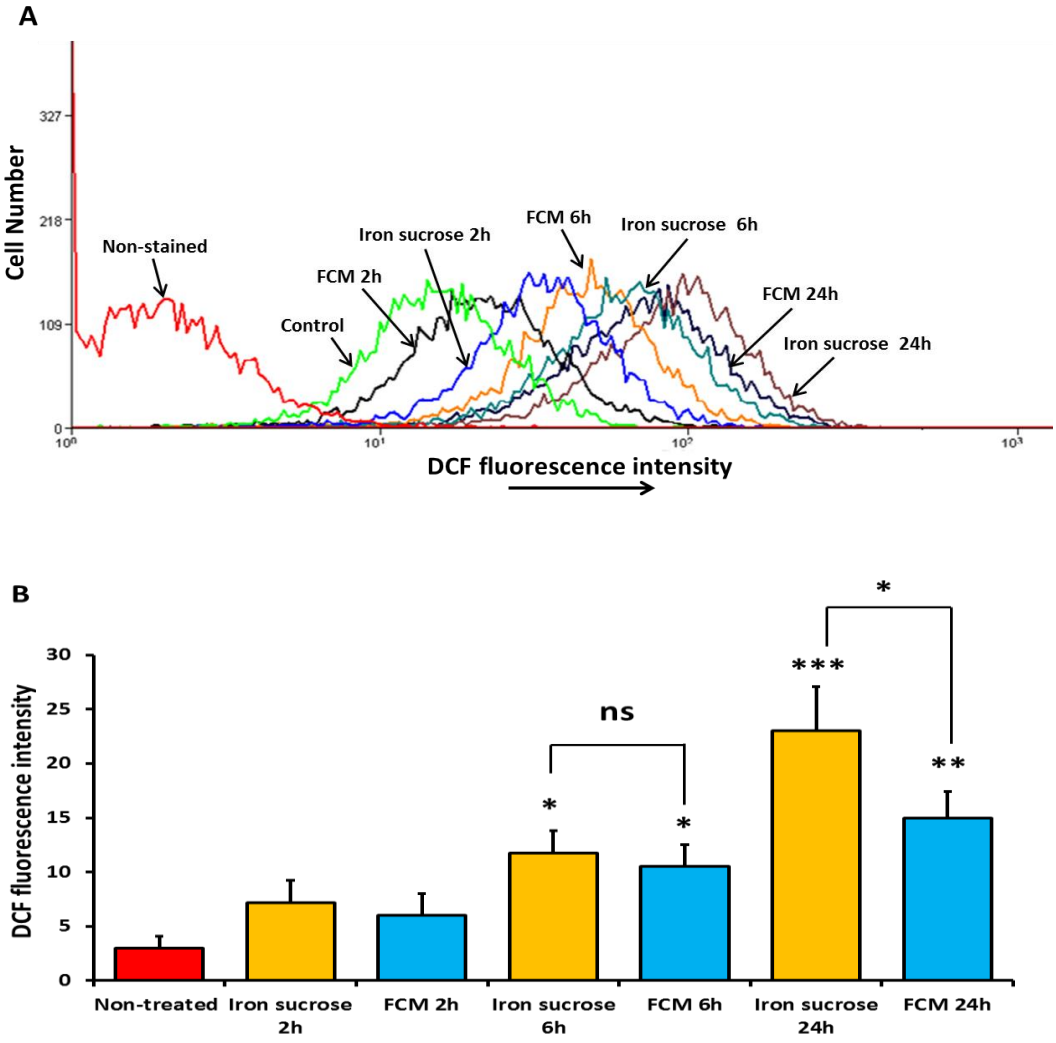


Figure 3.5: The effects of IV iron administrations on intracellular total ROS production in HUVECs using DCFH-DA by flow cytometry. HUVEC were grown in complete endothelial growth medium and either not treated or treated with 50 µg/ml IV iron sucrose or IV FCM for 2, 6 and 24hr. Non-treated were considered as negative controls. These cells were washed with DPBS then incubated with 10 µM DCFH-DA for 30-45 min. **(A):** representative flow cytometry histogram of HUVECs labelled with DCFH-DA. **(B):** mean fluorescence obtained from the histogram statistics. The fluorescence intensity of DCF was read at 525nm emission when excited at 488nm by flow cytometry. The results are expressed as mean (+/- SEM) from 3 different donors (n=3). *p<0.05, **p<0.01 and ***p<0.001 compared with non-treated cells and *p<0.05 for cells treated with IV iron sucrose compared with cells treated with IV FCM at 24hr.

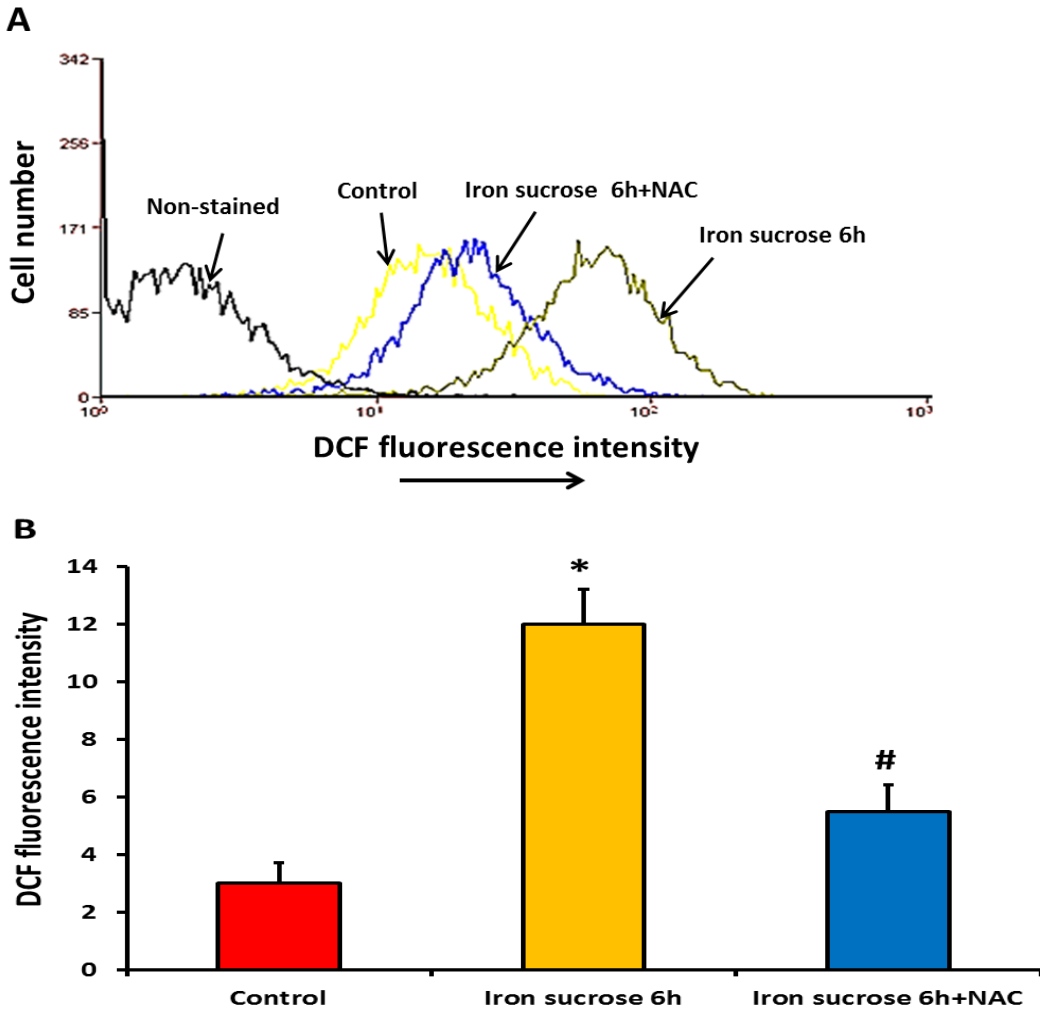


Figure 3.6: Representative images showing the effect of IV iron sucrose and NAC co-incubation on ROS generation in HUVECs using DCFH-DA by flow cytometry at 6hr. HUVEC were grown in complete endothelial growth medium and either not treated or treated with 50 µg/ml IV iron sucrose alone or in combination with 2 mM NAC for 6hr. Non-treated were considered as control. These cells were washed with DPBS then incubated with 10 µM DCFH-DA for 30-45 min. **(A):** representative flow cytometry histogram of HUVEC labelled with DCFH-DA. **(B):** quantitative analysis of the mean fluorescence intensity of DCF. The fluorescence intensity of DCF was read at 525nm emission when exited at 488nm by flow cytometry. The results are expressed as mean (+/- SEM) from 3 different donors (n=3). *p<0.05 compared with non-treated cells. $^{\#}p$<0.05 for cells treated with IV iron sucrose and NAC compared with HUVEC treated with IV iron sucrose at 6hr alone.

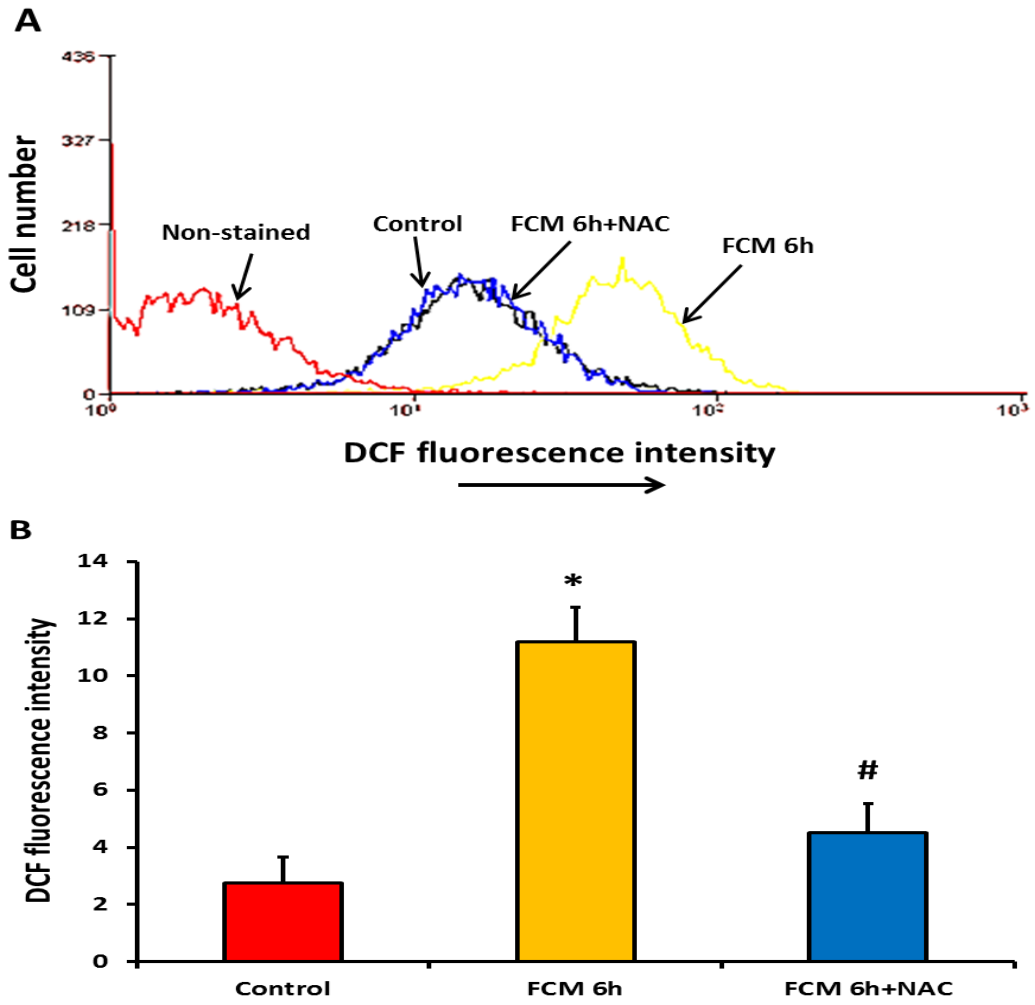


Figure 3.7: Representative images showing the effects of IV FCM and NAC co-incubation on ROS generation in HUVECs using DCFH-DA by flow cytometry at 6hr. HUVEC were grown in complete endothelial growth medium and either not treated or treated with 50 µg/ml IV FCM alone or in combination with 2 mM NAC for 6hr. Non-treated were considered as negative controls. These cells were washed with DPBS then incubated with 10 µM DCFH-DA for 30-45 min. **(A):** representative histogram of DCF intensity in HUVECs labelled with DCFH-DA and measured by flow cytometer. **(B):** mean fluorescence obtained from the histogram statistics. The fluorescence intensity of DCF was read at 525nm emission when exited at 488nm by flow cytometry. The results are expressed as mean (+/- SEM) from 3 different donors (n=3). **p*<0.05 compared to non-treated cells. $^{\#}p$<0.01 for HUVEC treated with IV FCM and NAC versus cells treated with IV FCM at 6hr alone.

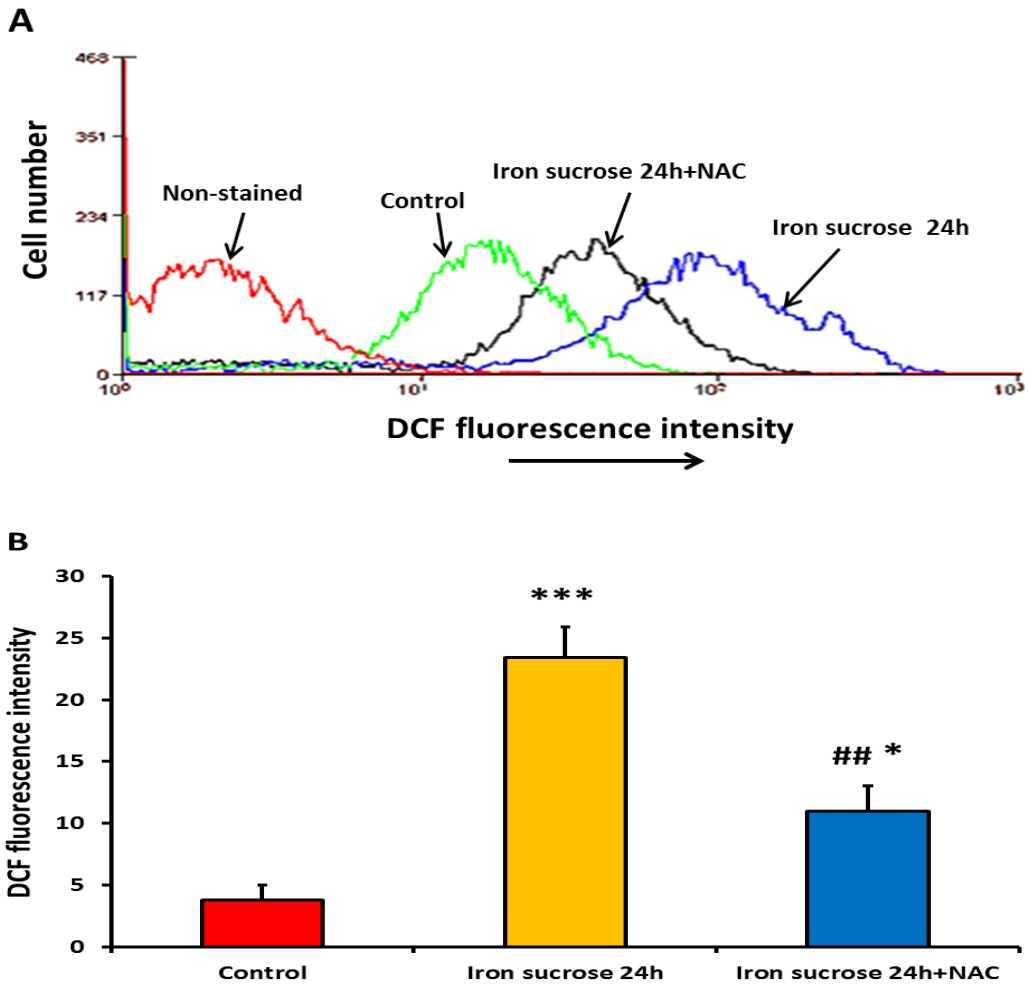


Figure 3.8: Representative images showing the effect of IV iron sucrose and NAC co-incubation on ROS generation in HUVECs using DCFH-DA by flow cytometry at 24hr. HUVEC were grown in complete endothelial growth medium and either not treated or treated with 50 µg/ml IV iron sucrose alone or in combination with 2 mM NAC for 24hr. Non-treated were considered as negative controls. These cells were washed with DPBS then incubated with 10 µM DCFH-DA for 30-45 min. **(A):** representative flow cytometry histogram of HUVEC labelled with DCFH-DA. **(B):** mean fluorescence obtained from the histogram statistics. The fluorescence intensity of DCF was read at 525nm emission when exited at 488nm by flow cytometry. The results are expressed as mean (+/- SEM) from 3 different donors (n=3). ***p<0.05 versus non-treated cells, $^{\#\#}p$<0.01 for HUVEC treated with IV iron sucrose and NAC compared with IV iron sucrose-treated cells alone and *p<0.05 IV iron sucrose and NAC together versus non-treated cells.

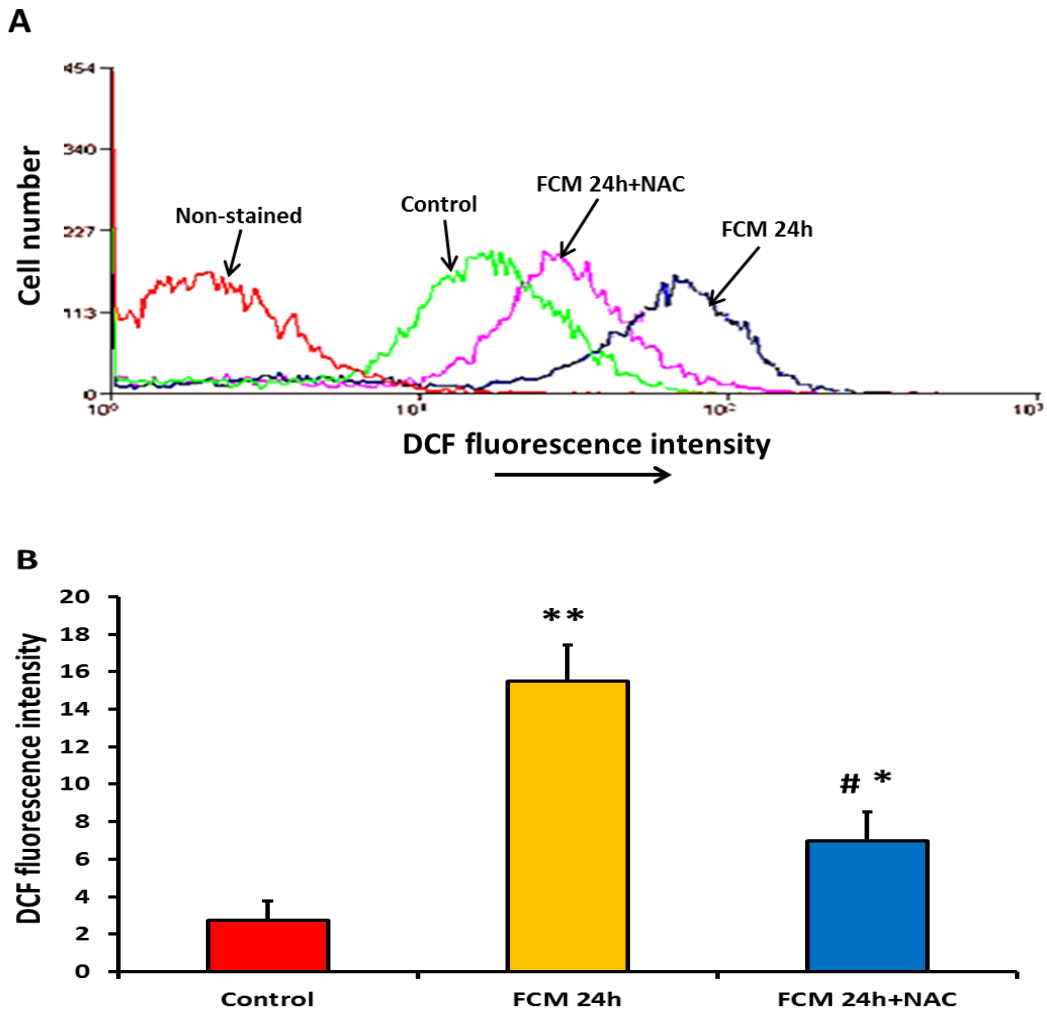


Figure 3.9: Representative images showing the effect of IV FCM and NAC co-incubation on ROS generation in HUVECs using DCFH-DA by flow cytometry at 24hr. HUVEC were grown in complete endothelial growth medium and either not treated or treated with 50µg/ml IV FCM alone or in combination with NAC (2mM) for 24hr. Non-treated were considered as control. These cells were washed with DPBS then incubated with 10µM DCFH-DA for 30-45min. **(A):** representative flow cytometry histogram of HUVEC labelled with DCFH-DA. **(B):** mean fluorescence obtained from the histogram statistics. The fluorescence intensity of DCF was read at 525nm emission when exited at 488nm by flow cytometry. The results are expressed as mean (+/- SEM) from 3 different donors (n=3). ***p*<0.05 versus non-treated cells, $^{\#}p$<0.01 for IV FCM and NAC-treated cells versus IV FCM-treated HUVEC alone and **p*<0.05 for IV cells treated with IV FCM and NAC versus non-treated cells.

3.3.3. Intracellular superoxide anion measurement in HUVEC treated with IV iron preparation and NAC using NBT assay

While DCF measure all ROS (including NO), the NBT assay specifically measures superoxide anion generation. Similar to the results obtained from total ROS production by DCFH-DA staining, Figure 3.10 showed clear detectable production of superoxide anion in non-treated cells. Although, IV iron sucrose or IV FCM at 2hr show increased superoxide anion production compared to non-treated cells, this was not statistically significant (Figure 3.10). In contrast, IV iron sucrose or IV FCM at 6 and 24hr incubation show clearly detectable production in superoxide anion, which were significantly increased compared to non-treated cells ($p<0.05$), ($p<0.01$) and ($p<0.001$) (Figure 3.10). In addition, HUVEC treated with IV iron sucrose showed significant increase compared with IV FCM-treated cells at 24hr ($p<0.05$) (Figure 3.10). Co-treatment with 2 mM NAC and IV iron sucrose or IV FCM resulted in significant reduction of superoxide anion compared with HUVEC treated with IV iron sucrose or IV FCM alone at 6hr and 24hr ($p<0.05$) and ($p<0.01$) respectively (Figure 3.11).

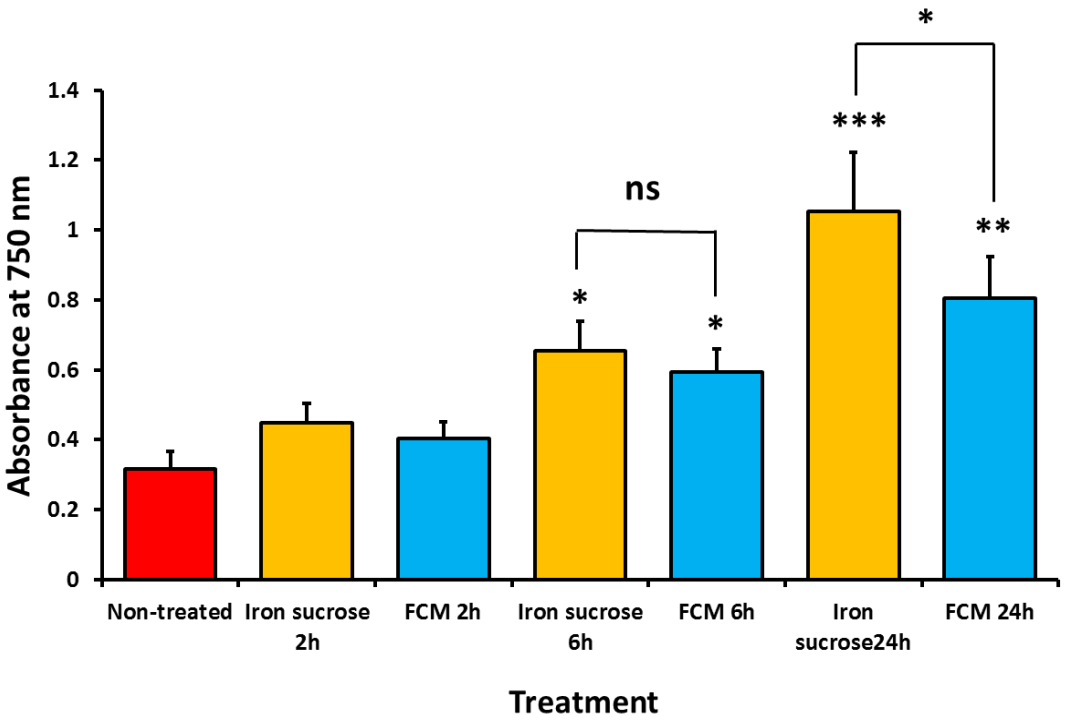


Figure 3.10: The effect of IV iron preparations on superoxide anion production in HUVEC using NBT assay**.** HUVEC were grown in complete medium and either not treated or treated with 50 µg/ml IV iron sucrose or IV FCM for different time course 2, 6 and 24hr. Non-treated cells from the same donor were considered as negative control. Media was replaced with phenol red-free media containing 1 mg/ml NBT and incubated at 37^0C in 5% CO_2 for 90 min. Cells were then lysed and absorbance was measured immediately with a spectrophotometer at wavelength 750nm as described in (Section 3.2.2.2.3). The results are expressed as mean (+/- SEM) from 6 different donors (n=6). *p<0.05, **p<0.01 and ***p<0.01 versus non-treated cells. *p<0.05 for HUVEC treated with IV iron sucrose at 24hr versus IV FCM-treated cells at 24hr.

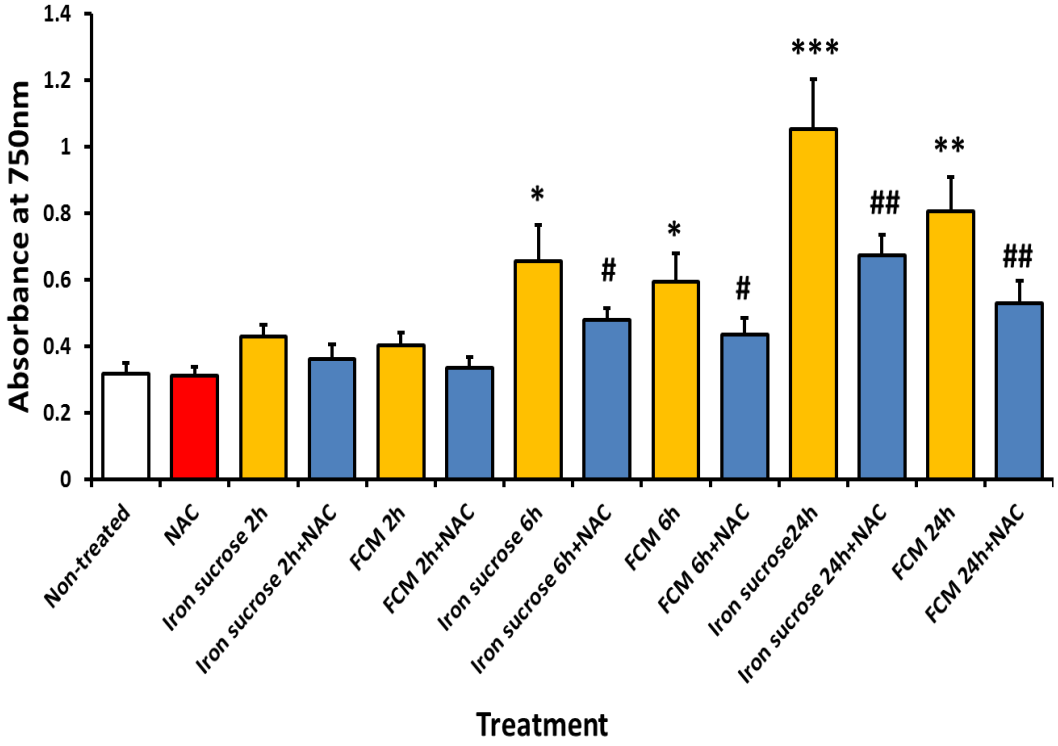


Figure 3.11: The effect of IV iron preparations and NAC co-incubation on superoxide anion production in HUVEC using NBT assay. HUVEC were grown in complete medium and wither not treated or treated with 50 µg/ml IV iron sucrose or IV FCM alone or with 50 µg/ml IV iron sucrose or IV FCM and 2 mM NAC for different time course 2, 6 and 24hr. Non-treated cells were considered as negative control. Media was replaced with phenol red-free media containing 1 mg/ml NBT and incubated at 37^0C in 5% CO_2 for 90 min. Cells were then lysed and absorbance was measured immediately with a spectrophotometer at wave length 750nm as described in section (3.2.2.2.3). The results are expressed as mean (+/- SEM) from 4 different donors (n=4). *p<0.05, **p<0.01 and ***p<0.01 versus non-treated cells. $^{\#}p$<0.05 for IV iron sucrose or IV FCM at 6hr compared with IV iron sucrose or IV FCM and NAC treated cells at 6hr and $^{\#\#}p$<0.05 for IV iron sucrose or IV FCM at 24hr compared with IV iron sucrose or IV FCM and NAC treated cells at 24hr.

3.4. DISCUSSION

This chapter provides experimental support for the concept that IV iron administration can trigger oxidative stress, by increasing ROS production which may lead to endothelial injury, a factor that plays a role in development of CVD in CKD patients (Lahera *et al*., 2006). There are several different methods used in the literature to investigate and measure ROS production in the cells (Tarpey and Fridovich, 2001). Even with several methods available, vascular ROS formation is not easy to measure, as the cytoplasmic and mitochondrial SOD enzyme scavenges the intracellular low steady state of superoxide anion, which can leak to the extracellular spaces (Rosen and Freeman, 1984). Thus, efficient and sensitive methods are needed in order to measure the relatively short half-life of ROS (Tarpey and Fridovich, 2001). In this study, to measure both total ROS production in EA.hy926 endothelial cell line and primary HUVEC, and superoxide anion in HUVEC, cells were exposed to IV iron sucrose or IV FCM, and two different methods were preformed, the DCFH-DA staining and NBT reduction assay.

ROS in the cells can oxidize DCFH-DA into its fluorescent derivative DCF. Numerous studies have shown that different types of ROS such as H_2O_2 or hydroxyl radical can oxidize DCFH-DA to DCF in BAEC (Royall and Ischiropoulos, 1993; Tampo *et al*., 2003), HUVEC (Hsieh *et al*., 1998) and by oxidants produced during the respiratory burst in inflammatory cells (Tarpey and Fridovich, 2001). However, Kalyanaraman *et al* (2011) have reviewed that DCFH-DA staining can not be used to measure H_2O_2 directly and this is due to its complexity and a number of limitation associated with this staining such as, cytochrome c have the ability either directly or indirectly to oxidize

DCFH to DCF (Karlsson *et al*., 2010; Burkitt and Wardman, 2001). Iron in its active state Fe^{2+} can promote the oxidation of DCFH to DCF in the presence of H_2O_2 (Qian and Buettner, 1999). However, all these limitations, can lead to incorrect fluorescence intensity of DCF.

In order, to confirm our data obtained by DCFH-DA staining and to determine if superoxide anion is elevated by IV iron preparations in HUVEC, NBT assay was used. The data of flow cytometry using DCFH-DA staining showed that total ROS production was significantly induced in EA.hy926 cell line and primary HUVEC treated with for IV iron sucrose or IV FCM compared with non-treated cells (Figures 3.2B, 3.3B, 3.4B and 3.5B). This indicates that both IV iron supplement induced an increase in the generation of total intracellular ROS production in both primary HUVEC and EA.hy926 cell line. However, HUVEC seemed to be more sensitive to IV iron preparations compared with EA.hy926 cell line, as our data showed that EA.hy926 cell line exposed to up to 500 µg/ml of IV iron sucrose or IV FCM, and cells did not die and no sign of cell death such as shrinkage was observed under the microscope compared with non-treated cells. While, our data from chapter 2, showed that HUVEC treated with 50 µg/ml of IV iron sucrose significantly reduced cell proliferation, obvious morphological changes and cells became shrunk and star-shaped with sharp outlines sign of apoptosis compared with non-treated cells. These data are indicative that EA.hy926 cell line may have better antioxidant defence system than primary HUVEC, although non-treated EA.hy926 cell line showed to generate more ROS compared with non-treated HUVEC.

Our data can be interpreted as being in line with Gupta *et al* (2010), who also used DCFH-DA staining and found that, following exposure of peripheral blood mononuclear cells (PBMC) to 100 µg/ml of IV iron sucrose, iron dextran and sodium ferric gluconate for 24hr, all three types of IV iron preparations significantly increased total intracellular ROS level in these cells. However, the concentration of IV iron preparations that was used in our study was half of what Gupta *et al* have used which suggests that HUVEC are more sensitive to those compounds. Kuo *et al* (2008) also found that, ROS was significantly induced in lymphocyte treated with serum from CKD patients who were treated with a range from 200 to 500 mg/ml IV iron sucrose using DCFH-DA staining analysed by flow cytometry. The authors assumed that this was as a result of antioxidant reduction since they found that plasma level of vitamin C and E were significantly reduced in the CKD patients. In addition, a recent *in vitro* study by Martin-Malo *et al* (2012), found that iron dextran, IV iron sucrose, IV FCM, and ferric gluconate significantly increased ROS production in haemodialysis patients. Martin-Malo *and* colleagues found that treating PBMCs from healthy donors and CKD patients on dialysis with 200 µg/ml IV iron sucrose, IV FCM, iron dextran and ferric gluconate for 2, 8 and 24hr, significantly induced total ROS production at 8 and 24hr compared to non-treated PBMC (Martin-Malo *et al*., 2012). These authors propose that ROS increased due to the NTBI released from each IV iron preparation. A more recent *in vitro* study by Fell *et al* (2014) treated PBMCs with 133, 266 and 533 µg/mL IV iron sucrose or IV FCM for 3hr. Fell *and* others used carboxy- H_2DFFDA staining by flow cytometry to measure ROS production and found that IV iron sucrose significantly increases ROS

production in these cells, while IV FCM had no significant effect. Because only IV iron sucrose increases ROS production, these authors suggested that this can be due to the pharmacokinetic differences between the two compounds as discussed in section 1.6.3.1. All these differences can play a key role in free iron released from IV iron preparations, which then leads to the induction of ROS production (Fell *et al*., 2014). However, the data of Fell *and* others study can be criticized as they incubated the cells with IV iron preparations only for 3hr, which could bias their results as ROS production by IV FCM may be evident after longer incubation period. In contrast, *in vivo* study by Malindretos *et al* (2007) showed that treating 20 CKD patients with 100 mg IV iron sucrose and iron dextran has no effect on oxidative stress levels. Another recent *in vivo* study showed that, treating anaemic mouse model with IV FCM, no significant effects on oxidative stress induction were observed (Heming *et al*., 2012).

Many reports in the literature have demonstrated the beneficial effect of NAC in the treatment of CKD patients. An *in vivo* study by Hsu *et al* showed that treating CKD patients with NAC improved their anaemia and decreased the plasma level of oxidized LDL (Hsu *et al*., 2010). In addition, another study on 134 CKD patients who were treated with 600 mg NAC twice a day for a week, has shown significant reduction in cardiac events and ischaemic stroke by 30% and 36% respectively (Tepel *et al*., 2003). Other studies have documented the beneficial effect of NAC on improvement of endothelial function in CKD patients (Scholze *et al*., 2004; Libetta *et al.*, 2011). To explore whether antioxidant treatment can reduce or normalize the excess level of ROS production, *in vitro* HUVEC were treated with 2 mM NAC and

ROS levels measured using NBT reduction assay and DCFH-DA staining. NAC reduced the ROS inducing effect of IV iron sucrose or IV FCM. This reduction was significant (p<0.05) for IV iron sucrose or IV FCM and NAC at 6 and 24hr compared to IV iron sucrose or IV FCM at 6h and 24hr alone, which indicates that NAC was an efficient ROS scavenger and was able to attenuate IV iron preparation-induced ROS production in HUVEC. Our results can be in line with an *in vivo* study where MDA was looked at. Agarwal *et al* (2004) and Swarnalatha *et al*., (2010), who found that treating CKD patients with 100 mg of IV iron sucrose and 600 mg NAC, significantly reduced the amount of plasma MDA that was elevated by IV iron sucrose in these patients. Therefore, both our *in vitro* data and published *in vivo* data indicate that NAC is effective in reducing oxidative stress levels.

Several studies have been previously published on the measurement of superoxide anion by NBT reduction assay (Au-Yeung *et al*., 2004; Eligini *et al*., 2005; Patterson *et al*., 1999). Superoxide anions reduce NBT to monoformazan (NBT^{+}) which can be detected spectrophotometrically. From our data in this chapter, both treated and non-treated cells showed detectable production of superoxide anion. These results could be interpreted as being in line with other studies which documented that ECs produce low levels of superoxide anion as a second messenger which play an important role in mediating different signalling pathways under physiological conditions (Kunsch and Medford, 1999; Ullrich and Bachschmid, 2000; Zhang and Gutterman, 2007). However, IV iron-treated HUVEC showed increases superoxide anion production compared with non-treated cells, and this increase was significant for cells that were treated with

either IV iron sucrose or IV FCM at 6hr incubation compared with non-treated cells (p<0.05) and for IV iron sucrose or IV FCM at 24hr incubation compared with non-treated cells (p<0.01). Even though both IV iron preparations induce superoxide anion this was not to a similar extent at different time points. IV iron sucrose at 24hr showed greater capacity in generating superoxide anion than IV FCM at the same time point. Our result are consistent with the finding by Rooyakkers *and* others who showed 100 mg/ml IV iron sucrose significantly induced superoxide anion production by 53-70% in healthy volunteers after 10 and 240 min of administration (Rooyakkers *et al*., 2002). These group suggest that increase of superoxide anion, was due to NTBI or free iron, which acts as a catalytic agent in the formation of oxygen radicals. Also consistent with the finding by Kuo *et al*., who showed that superoxide anion was significantly increased in CKD patients treated with 800 mg/l of IV ferric chloride hexahydrate. Kuo *and* others suggest that is because the high doses of IV iron which might increase the amount of NTBI or free iron, and subsequently superoxide production (Kuo *et al*., 2012). This indicates that IV iron preparation not only increase oxidative stress in CKD patients but also in healthy people.

Collectively, all these findings indicate that IV iron preparations can lead to EC dysfunction and through overproduction of ROS generation. It is well known that, normal function of EC is to produce NO, and because EC dysfunction is associated with reduction of NO. Therefore, EC dysfunction as a result of IV iron preparation treatment can be due to decreased tissue bioavailability of NO. Together, our data obtained by both the NBT reduction assay and DCFH-DA probe give solid evidence that treatment with IV iron

preparations induced oxidative stress in HUVEC. This increased ROS could explain, at least partially the inhibition of ECs proliferation rate and the marked changes in HUVEC morphology by IV iron sucrose. In addition, IV FCM also showed to induce ROS generation compared with non-treated cells. However, IV FCM did not generate as much ROS as the IV iron sucrose and may explain our data from chapter 2, where IV FCM did not promote EC dysfunction compared with non-treated cells. Thus, the findings of this study suggest that increased ROS generation and superoxide anion induced by IV iron preparations may play a major role in EC dysfunction and potentially provide a fundamental mechanism linking the high prevalence of CVD in CKD patients.

3.5. CONCLUSION

In conclusion, taken together, these results indicate that IV iron sucrose or IV FCM exposure induces total intracellular ROS generation. This study documents for the first time that IV FCM compound induced superoxide anion production in EC. Oxidative stress, in turn can result in EC damage and, subsequently CVD in CKD patients. IV iron sucrose is more potent in generating superoxide and total ROS when compared to IV FCM. In addition, treatment with NAC has clear evidence in protecting EC from oxidative stress, which was induced by the selected IV iron preparations.

CHAPTER 4

EFFECT OF IV IRON PREPARATIONS ON HUVEC DAMAGE

4.1. INTRODUCTION

Apoptosis or programmed cell death is an essential part of normal development, and plays a role in homeostasis to control cell populations in tissue (Elmore, 2007). Apoptosis also works as a defence mechanism for instance, in the induction and generation of regulatory T- lymphocyte cells, which play a key role in regulating the immune system in times of infection to get rid of harmed cells (Norbury and Hickson, 2001). However, inappropriate activation of apoptosis may contribute to a variety of clinical disorders, including those that affect the vascular system such as atherosclerosis (Van Vré *et al*., 2012). ROS which function as regulators of subcellular events, when overexpressed can also induce DNA, lipid, and protein damage and cell death through intrinsic and extrinsic apoptotic pathways, suggesting a link between ROS and apoptosis (Wen *et al*., 2002; Uttara *et al*., 2009). ROS are known to induce apoptotic cell death in different cell types, including ECs (Dimmeler *et al*., 2000). Furthermore, in the cardiovascular system, apoptotic cell death following injury of vascular endothelium may play a role in initiation of atherogenesis as well as in the induction of acute events which trigger myocardial infarction (Cines *et al*., 1998; Dimmeler *et al*., 1997; Falk, 2006; Rudijanto, 2007). Excessive ROS production includes $O_2^{\cdot -}$ and H_2O_2, can cause apoptosis in ECs (Irani, 2000; Li and Shah, 2004). EC apoptosis results in increased vascular permeability to cells (leukocyte) and lipids, smooth muscle cell proliferation, and increased coagulation, thus contributing to the development of atherosclerotic lesions (Pirillo *et al*., 2013). IV iron administration has been frequently used for the treatment of iron deficiency and for the management of anaemia in CKD as previously discussed

(Kamanna *et al*., 2012). However, several *in vitro* and *in vivo* studies showed that these compounds, in particular IV iron sucrose promote apoptosis in different cell types including, ECs and lymphocytes (Kartikasari *et al*., 2006; Carlini *et al*., 2006; Ichii *et al*., 2012; Martin-Malo et al., 2012; Kamanna *et al*., 2012; Masuda et al., 2013). Furthermore, previous *in vitro* studies showed that IV iron sucrose causes endothelial damage through oxidative stress induction (Zager *et al*., 2002; Bishu and Agarwal, 2006; Kamanna *et al*., 2012).

Enzymes within the p38 MAPK family are widely distributed among different tissues and play an important role in regulating many cellular processes such as differentiation, cell growth, death and DNA repair and can also contribute to the regulation of cell apoptosis upon stress stimuli (Ono and Han, 2000; Tian *et al*., 2012). Elevated ROS has shown to directly injure the cells by oxidizing DNA and protein (Klaunig *et al*., 2010). However, different studies showed that high level of ROS can also damage the cells indirectly by activating multiple signalling pathways, including MAPK cascades, p38 MAPK in different tissue and in different arteries ECs from human (HAEC), rat (RAECs) and mouse (MAECs), and are often associated with apoptosis (Callsen and Brune, 1999; Osone *et al*., 2004; Sigaud *et al*., 2005; Tian *et al*., 2012). Studies have shown that apoptosis can be mediated by p38 MAPK in EC (EA.hy926 cell line) in response to TNF-α (Yue *et al*., 1999; Grethe *et al*., 2004). Other studies have described the anti-apoptotic effects of this pathway as protective on cardiac myocytes (Zechner *et al*., 1998). It has been shown that, many death and survival genes, including Bax and Bcl-2, which are located on the mitochondria can alter mitochondrial membrane

permeability and trigger apoptosis (Yuan *et al*., 2003). The expression of Bax is regulated by redox-sensitive transcription factor NF-kB, and oxidative stress increases Bax expression through the activation of NF-kB producing a pro-apoptotic state (Shou *et al*., 2002; Wiu *et al*., 2001). P38 MAPK activation has been shown to phosphorylate Bcl-2 and lead to its translocation out of the mitochondria, which seem to be essential for the activation of the intrinsic pathway apoptotic signal, leading to release of cytochrome *c* and downstream activation of caspase-3, resulting in apoptosis (Farley *et al*., 2006). Furthermore, in a cardiac myocyte cell line, Bax protein and mRNA gene expressions were also shown to be up-regulated by p38α MAPK, which resulted in cell apoptosis (Porras *et al*., 2004). Oxidative stress including 200 μM H_2O_2, induced apoptosis in rat cardiac myocyte, due to significant down-regulation of Bcl-2 protein expression. This down-regulation of Bcl-2 was attenuated by p38 MAPK inhibitor, SB203580 (Markou *et al*., 2009), which indicates that p38 MAPK plays an important role in down regulation of Bcl-2 protein and apoptosis. In addition, p38 MAPK was shown to play a key role in CVD such as ischaemia reperfusion injury, through the induction of apoptosis, via the modulation of Bcl-2 family proteins (Kaiser *et al*., 2004).

Chapter 2 and 3 indicated clearly that, IV iron preparations, in particular IV iron sucrose significantly inhibited proliferation and induced ROS generation in ECs. In this chapter, we hypothesise that ROS generation by IV iron preparations can mediate the activation of p38 MAPK and consequently lead to both down-regulation of Bcl-2 and up-regulation of Bax, leading to apoptosis in HUVEC. Therefore, this chapter aimed to investigate the *in vitro*

effect of IV iron sucrose or IV FCM on induction of EC apoptosis using Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL assay) and Annexin V-FITC/PI. In addition, the work aimed to demonstrate whether treatment with these two compounds induce sustained activation of p38 MAPK via ROS elevation and whether this is responsible for the down-regulation of Bcl-2 and up-regulation of Bax protein expressions, as two key anti and pro-apoptotic proteins involved in stress-induced apoptosis pathway, leading to apoptosis of ECs using Western blot method. The role of p38 MAPK in apoptosis was investigated using the specific inhibitor, SB203580 on IV iron preparation-induced apoptosis to confirm the role of p38 MAP kinase pathway in apoptosis.

4.2. MATERIALS AND METHODS

4.2.1. Materials

4.2.1.1. Reagents

Sodium chloride was purchased from Sigma-Aldrich. Sodium dodecyl sulphate (SDS, L3771), glycine (G-8898), sodium pyrophosphate (S-9515), bromophenol blue (B-5525), *N,N,N',N'*- tetramethylethylenediamine (TEMED, T-9281), Dl-dithiotheritol (D-9163), ammonium persulfate (APS, A-3678), Kodak GBX developer and replenisher (P7167) and Kodak fixer and replenisher (P7042), Kodak X-ray film (F1274-50). HEPES (H4034) were purchased from Sigma-Aldrich Chemical Company (Pool Dorest, UK). Polyvinylidene difluoride (PVDF) membrane (Hypond-P, RPN303F) was purchased from GE healthcare UK limited, Buckinghamshire, UK. Acrylamide/bis solution (30%) (161.0156), protein assay kits (500-0120) were obtained from BioRad (Bio-Rad Laboratories, Hercules, California, USA). All reagents for enhanced chemiluminescence (ECL) were purchased from Amersham Life sciences (Little Chalfont, Bucks, UK). P38 MAP kinase inhibitor SB203580 (ab120162) was obtained from Abcam (Cambridge, UK). Terminal Deoxynucleotidyl Transferase, Recombinant (M1871) was obtained from Promega (Southampton, UK). Annexin V-FITC/PI Apoptosis Detection Kit (ab14085) was purchased from Abcam (Cambridge, UK). Phosphate buffered saline (PBS) pH 7.4.

4.2.1.2. Antibodies

Rabbit polyclonal IgG phospho-p38 MAP Kinase (MAPK) (Thr180/Thr182) antibody (9212S), Bcl-2 Antibody (Human Specific) (2872) and Bax Antibody (2772) were purchased from New England Biolabs, UK. ECL anti- rabbit IgG horseradish peroxidise HRP linked whole antibody (NA934V) was purchased from GE healthcare UK limited, Buckinghamshire, UK. Biotinylated protein ladder (catalog No. 7727) and HRP conjugated anti biotin antibodies (catalog No. 7075) were purchased from New England Biolabs, UK. Prestained Protein Ladder (catalog No. 26616) was purchased from Thermo Fisher Scientific, UK. The diluent which has been used to dilute all antibodies was TBS-T including, 2% w/v BSA. Glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH) (ab9485) was purchased from Abcam (Cambridge, UK).

4.2.2. Methods

4.2.2.1. Determination of apoptosis in IV iron preparation treated-HUVEC

4.2.2.1.1. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL assay)

TUNEL assay was used to demonstrate whether IV iron sucrose or IV FCM induces apoptosis in cultured HUVEC. Gavrieli *and* colleagues (1992) have described TUNEL as an assay for localization of apoptotic DNA fragmentation (Gavrieli *et al*., 1992). The method relies on the template-independent identification of blunt ends of double-stranded DNA breaks, which can be recognised by TdT enzyme to measure the fragmented DNA of

apoptotic cells by catalytically incorporating fluorescein-16-dUTP (a) at 3´-OH DNA ends (Kyrylkova *et al*., 2012), as shown in (Figure 4.1).

HUVEC were grown onto coverslip slides in 6 well plates at a concentration of $2x10^5$ cells per well, treated with 50 µg/ml IV iron sucrose or IV FCM, and incubated at 37^0C in 5% CO_2 for 2, 6 and 24hr. Cells were then fixed in 4% paraformaldehyde in PBS pH 7.4 for 15 min at room temperature. Then slides were washed twice with PBS. Apoptosis was determined using a terminal TUNEL assay kit (Promega, Southampton, UK) according to the manufacture's protocol. Slides were incubated in a reaction mixture (30 mM Tris pH 7.4; 140 mM sodium cacodylate; 1 mM cobalt chloride; 5 µM biotin-16-deoxyuridine triphosphate; 0.3 U/µl terminal deoxynucleotidyl transferase (TdT) at 37^0C for 1hr, and then were washed twice in PBS. Additionally, cells were treated with extravidin peroxidase solution for 30 min in a moisture chamber at 37^0C. Slides were then washed in PBS and the chromogenic reaction visualized by the addition of 3, 3'-diaminobenzidine (DAB) for 8-10 min at room temperature, and contrastained with haematoxylin and coverslips was applied using Histomount mounting medium (Fisher Scientific, Fair Lawn, NJ) for light microscopic observation. Imaging and analyses were performed by using Olympus CKX31 microscopy (Olympus, Southend-on-Sea, UK). Cells incubated without TdT mixture reaction were considered as negative control.

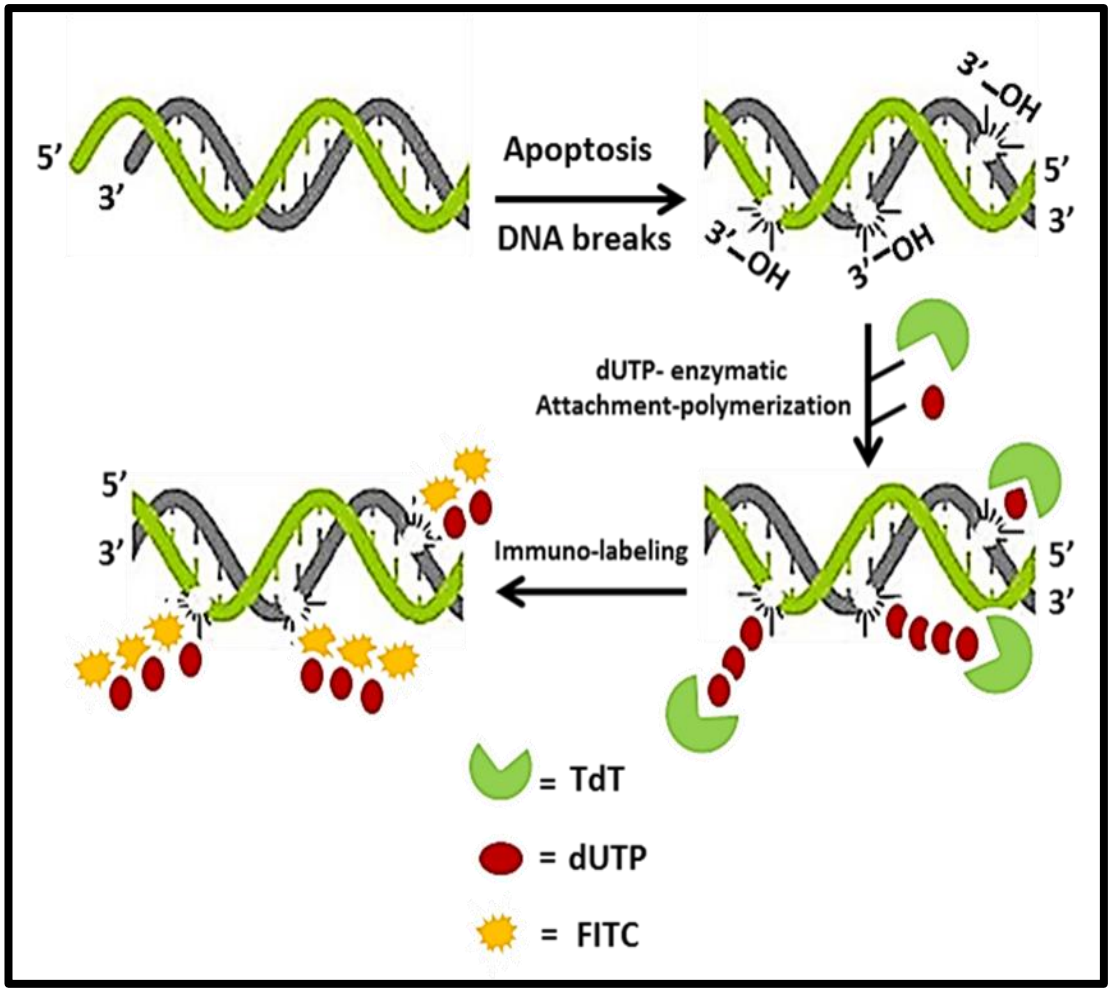


Figure 4.1: Schematic of the TUNEL assay. Staining of single or double stranded DNA breaks labelling with dUTP utilizing exogenous TDT enzyme and analysed under microscope.

4.2.2.1.2. Cell analysis for TUNEL assay

HUVEC were assessed for morphology and staining according to Gold *et al*., (1994). The following findings were considered to represent apoptosis: marked condensation of chromatin and cytoplasm clearly staining strongly brown or brown/black; the TUNEL-positive cells were scored in several fields on each coverslip to yield a total of at least 100 cells per field (10 fields in each slide) under a 40x objective of an Olympus CKX31 microscope. Values

represent percentages from at least 100 cells from each culture. Using the one-way ANOVA test differences were considered as significant at $p<0.05$.

4.2.2.1.3. Annexin V-FITC/PI staining method to detect apoptosis

Phosphatidylserine (PS) is plasma phospholipid membrane component located inside the cell membrane envelope (Lee *et al*., 2013). However, on induction of apoptosis, PS translocate to the outer surface of the cell, acting as signal for macrophages to overwhelm the cell (Lee *et al*., 2013). Thus, Annexin V-FITC/PI staining is a recombinant phosphatidylserine-binding protein that interacts strongly with phosphatidylserine residues and can be used for the detection of apoptosis as shown in Figure 4.2 (Schutte *et al*., 1998; Arur *et al*., 2003). Annexin V-FITC/PI apoptosis kit also contain propidium iodide (PI), which used to differentiate necrotic from apoptotic cells. PI is a membrane impermeant dye but once cells lose their membrane integrity, PI then enter and bind to nucleic acids of dead cells, which explain why PI-positive cells are necrotic as shown in Figure 4.2 (Riccardi and Nicoletti, 2006). HUVEC were grown in 6 well plates at a concentration of $1x10^5$ cells per well and incubated with 50 µg/ml IV iron sucrose or IV FCM for 24hr. Cells were trypsinised and washed with PBS, centrifuged at 13000x g for 5 min. Then, 1 $x10^{\mathbf{5}}$ cells from each well were suspended in 100 µl binding buffer (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$). Cells were then incubated with 5 µl Annexin V-FITC/PI and 5 µl of propidium iodide, and incubated for 15 min in the dark at room temperature. Subsequently, 500 µl binding buffer was added and the fluorescence of each sample was quantitatively analysed at 488nm by FACScalibur flow cytometry (Beckman Coulter, Inc. UK). Apoptosis was expressed as the percentage of

cells exhibiting Annexin V-binding relative to the total cell population (Rayner *et al*., 2006; Sun *et al*., 2007).

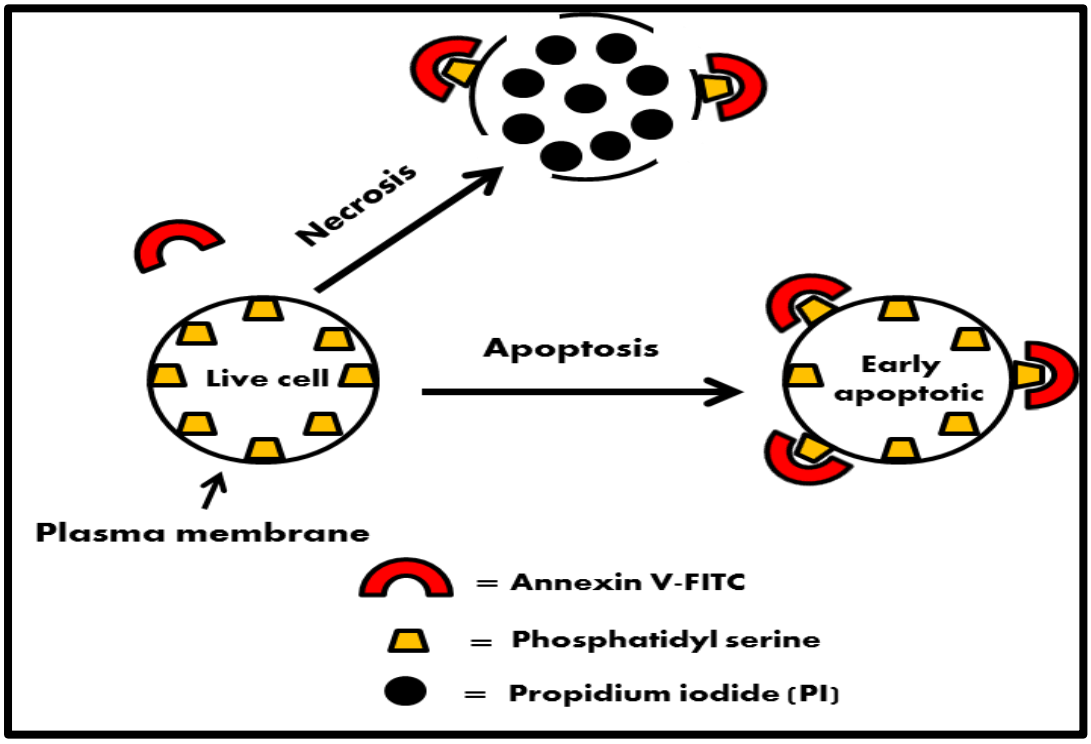


Figure 4.2: Schematic representation of apoptosis-induced membrane changes recognized by Annexin V-FITC/PI.

4.2.2.1.4. P38 MAP kinase role in IV iron preparation-mediated EC activation

4.2.2.1.5. Cell Treatment with IV iron preparations prior to Western blotting for phospho-p38 MAP Kinase, Bcl-2 and Bax

HUVEC were seeded in complete endothelial growth medium in 6 well plates at a concentration of 2 x10^5 cells per well, until they were 80-90% confluent. Cells were stimulated with 50 µg/ml of IV iron sucrose or IV FCM for 15 min, 30 min, 60 min, 2hr, 6hr and 24hr for detection of p38 MAPK, and 2, 6 and 24hr for Bcl-2 and Bax, and incubated in an atmosphere of 95% air and 5%

CO_2 at 37^0C. HUVEC left untreated were considered as negative control (0 µM).

4.2.2.1.6. Identification of phospho-p38 MAP Kinase, Bcl-2 and Bax by sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) and Western blotting

4.2.2.1.6.1. Preparation of samples for analysis by SDS PAGE

Following the appropriate stimulation, the 6 well plates were removed from the incubator at 37^0C and placed on ice. Media was aspirated from each plate, and the cells were washed twice with 500 µl ice-cold HPFEV buffer (50 mM Hepes, 10 mM sodium pyrophosphate $Na_4P_2O_7$, 100 µM sodium fluoride NaF, 4 mM EDTA and 2 mM orthovanadate Na_3Vo_4, pH7.4) to stop the reaction and inhibit protein dephosphorylation. Following buffer aspiration, adherent cells were harvested by scraping into 400 µl hot (65^0C) Laemmli sample buffer (0.048 M Tris-HCL, pH 6.8 containing 0.8 mM sodium pyrophosphate, 5 mM EDTA, 2% w/v SDS, 10% glycerol, 001% w/v bromophenol blue, 50 mM DTT) (Laemmli, 1970) and pulling the sample passed repeatedly through a 23G needle. The cells were then heated at 100^0C for 5 min in a heating block, in order to denature the protein present, and then stored at -20^0C until analysis.

4.2.2.1.6.2. Determination of protein concentration

The amount of protein in cell lysates was quantified to ensure equal loading in Western blot gels. Total protein in each HUVEC sample (without bromophenol blue) was analysed with Bradford protein assay kit (Bio-rad 500-0120) according to the manufacturer's instructions, with the detergent

compatible adaptation and the use of bovine serum albumin as standard (200, 400, 600, 800, and 1000 mg/ml). Initially, dilutions of the protein standard were prepared at above concentrations then 5 µl of the standard or cell lysate were added to individual wells of a 96 well-plate in triplicate. 25 µl of reagent A* (2% v/v reagent S in reagent A) was added to each well and then and then 200 µl of reagent B were added onto each well. The plates were left for 15 min at room temperature with gentle agitation to mix the reagents. The absorbance was read by 96 well plate reader at 750 nm, each sample producing a triplicate reading to allow the average of these triplicates to be calculated. The volume from each sample containing 30-40 µg of protein was then calculated and the appropriate volume of sample used for electrophoresis.

4.2.2.1.6.3. Resolution of protein by SDS-PAGE

A Bio-Rad mini protein gel electrophoresis kit (500-0120) was used to run all gels. Tris buffers were prepared and stored at room temperature for both resolving gel (1.5 M Tris base and 0.4% w/v SDS, pH 8.4) and stacking gel (0.5 M Tris base and 0.4% w/v SDS, pH 6.8). 10% resolving gel was prepared and poured in between glass plates and spacers (1.0 mm thick) to a level 1cm below the bottom of the comb when inserted and overlaid with 0.1% w/v SDS solution. Once the resolving gel polymerized, SDS was removed and a 3% stacking gel was poured on the top and a 1mm comb was inserted. Following gel polymerization, they were assembled and placed in a Prespex tank containing electrophoresis buffer (25 mM Tris base, 192 mM glycine and 0.1% w/v SDS). Appropriate volume from each sample containing 30-40 µg protein was loaded per well as well as 5 µl prestained

protein ladders (Thermo Fisher Scientific, UK) and biotinylated standards (New England Biolabs, UK). Samples were electrophoresed at a constant voltage of 100V for 2hr.

4.2.2.1.6.4. Western blotting

A Bio-Rad mini transfer Kit was used for the transfer of protein to PVDF membrane. PVDF membrane (GE healthcare, UK) was pre-wet in 100% methanol for 1-2 min and then washed with distilled water followed by blotting buffer (25 mM Tris base, 192 mM glycine and 20% v/v methanol) for 5 min each. A transfer sandwich was prepared containing black cassette, sponge, filter paper, resolving gel; pre wet PVDF membrane, filter paper and white cassette. This sandwich was inserted into a transfer tank buffer filled with blotting buffer. The whole tank was engulfed with ice and connected to the power supply at 100V for 2.30hr.

4.2.2.1.6.5. Detection of phospho-p38 MAPK, total p38 MAPK proteins, Bcl-2 and Bax

A number of different protocols were attempted to produce specific p-p38 MAPK, Bcl-2 and Bax bands as shown in Figure 4.3. Once transfer was completed, PVDF membrane was placed in blocking solution of either 3% w/v BSA or 5% w/v non-fat Milk in tris buffer saline-Tween (TBS-T) (150 mM sodium chloride, 20 mM Tris base pH7.4) containing detergent (Tween-20) at concentrations of 0.1% v/v overnight at 4^0C. The membrane was then incubated with p38 MAP kinase rabbit Ab (diluted in TBS-T,1:2000) or phospho-p38 MAP kinase rabbit mAb (diluted in TBS-T ,1:5000), anti-human Bcl-2 rabbit Ab (diluted in TBS-T,1:2000) or anti-Bax rabbit antibody (diluted

in TBS-T,1:2000) overnight with gentle agitation at 4^0C. The following day, the membranes were washed on a shaker 3 times for 10 min with 10ml TBS-T and then incubated with horseradish peroxidise HRP- conjugated donkey anti rabbit IgG (1:5000) for 1hr at room temperature with shaking. Membrane was washed as previously described then prepared for ECL detection.

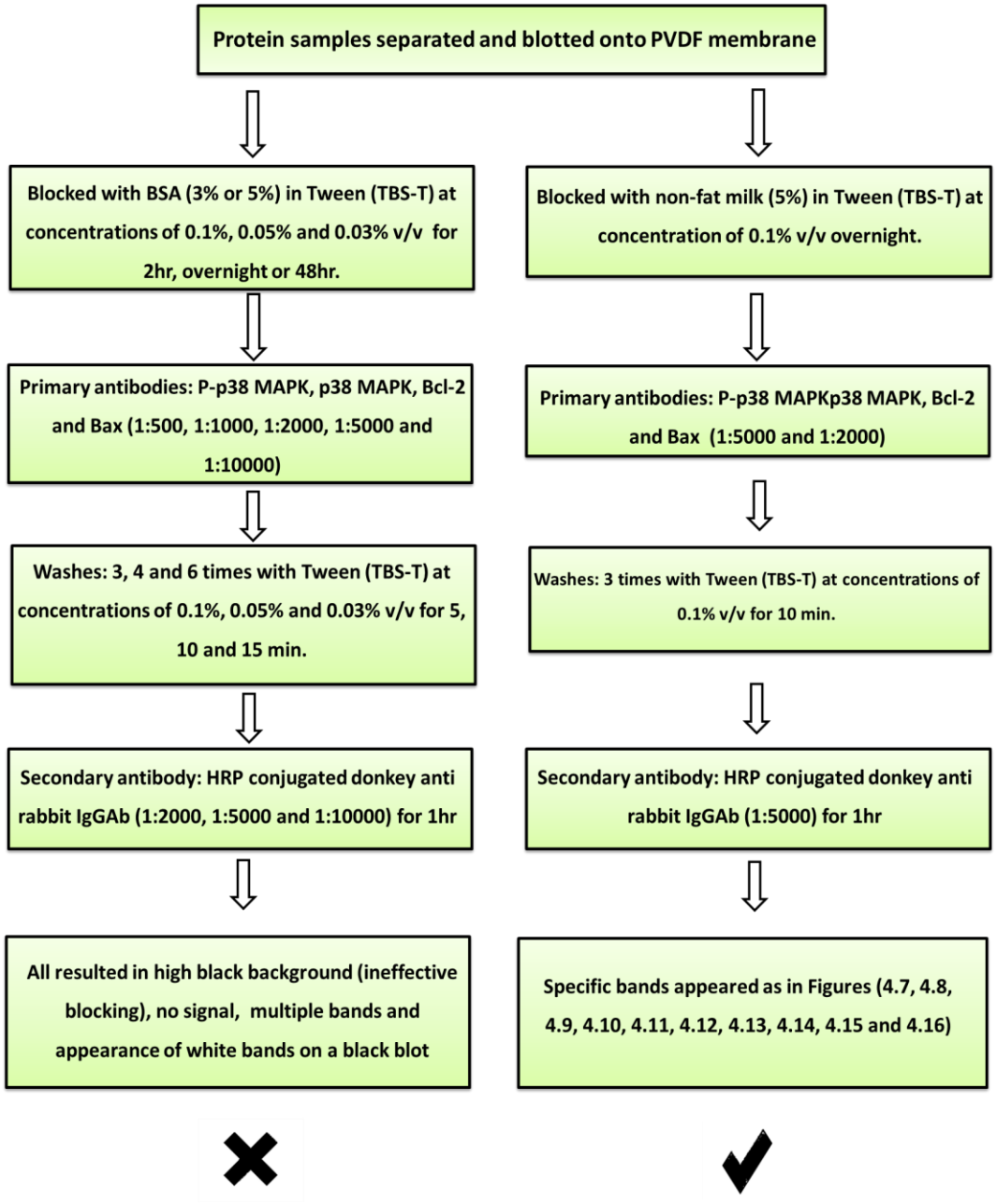


Figure 4.3: Flowchart representing developmental work that led to the final data of Western blot presented.

4.2.2.1.6.6. Detection of protein by enhanced chemiluminescence (ECL)

Following the final wash, the membrane was incubated in equal amounts of ECL solutions, ECL1 (250 mM luminol, 90 mM *p*-coumaric and 1 M tris base pH 8.5) and ECL 2 (30% H_2O_2 and 1 M tris base pH 8.5) for 3 min with gentle agitation and then placed in developing cassette, covered with saran wrap

excluding air bubbles. Kodak X-ray film was exposed to the membrane in the dark for 5 to 10 min. Film was immersed in developing solution for approximately 3 min until bands were visible, then it was washed with tap water before immersing it in fixing solution until the film became translucent. Film was rinsed thoroughly with tap water and left to air dry.

All Western blotting experiments were performed on at least 3 independent samples and densitometric analysis of ECL exposure was performed using ImageJ 1.48 software from NIH.

4.2.2.1.6.7. NAC treatment

The antioxidant NAC was used to investigate the role of ROS generation in p38 MAP kinase activation. HUVEC were pre-treated with 2 mM NAC and then cultured for 60 min and 2hr in complete endothelial cell growth medium containing 50 µg/ml IV iron sucrose or IV FCM. HUVEC from the same donors cultured for 3hr in complete endothelial growth medium alone or with 2 mM NAC and were considered as control.

4.2.2.1.6.8. P38 MAPK inhibition

SB203580, a specific inhibitor for p38 MAPK was used to inhibit the phosphorylation of p38 MAP kinase (Gum *et al*., 1998). HUVECs were cultured to reach 80-90% confluence. Cells were pre-treated with p38 MAPK inhibitor, SB203580 at a final concentration of 10 µM for 1hr. Then cells were treated with IV iron sucrose for 24hr. HUVEC treated with SB203580 alone were considered as a control.

4.3. RESULTS

4.3.1. Effects of iron preparations on the apoptosis of HUVEC

The effect of IV iron preparations treatment on HUVEC was evaluated by the TUNEL assay and apoptotic cells identified by a dark brown stain is confined to the nucleus. The results are expressed as percentages of apoptotic cells, as shown in Figure 4.4. TUNEL assay revealed that few cells treated with IV iron sucrose at 2 (6.2%) and 6hr (8.4%) respectively. Similarly treatment with IV FCM at 2 (5.9%) or 6hr (7.14%) respectively had undergone apoptosis and was not statistically significant compared with non-treated cells (5.2%) are shown in Figure 4.5. In contrast, exposure of cells to 50 µg/ml IV iron sucrose for 24hr showed a statistically significant increase in apoptotic cells compared with non-treated cells. The apoptosis of HUVEC treated with IV iron sucrose for 24hr increased to 38.5% when compared with non-treated cells (p<0.001) (Figure 4.4). Apoptotic cells showed dark brown nuclear staining indicative of DNA fragmentation associated with apoptosis. The results of the induction of apoptosis by IV iron preparations treatment of HUVEC are shown in Figure 4.4F. In contrast to iron sucrose, 24hr treatment with 50 µg/ml IV FCM did not cause any statistically significant increase in apoptosis compared to control HUVEC 9.2% (Figure 4.4G). TUNEL assay is somewhat subjective method and examination of HUVEC apoptosis was not performed by blinded observers. Therefore, a more quantitative method was also used. Annexin V-FITC/PI by flow cytometry as described in section (4.2.2.1.3) in order to confirm our results by TUNEL assay, which gave similar results.

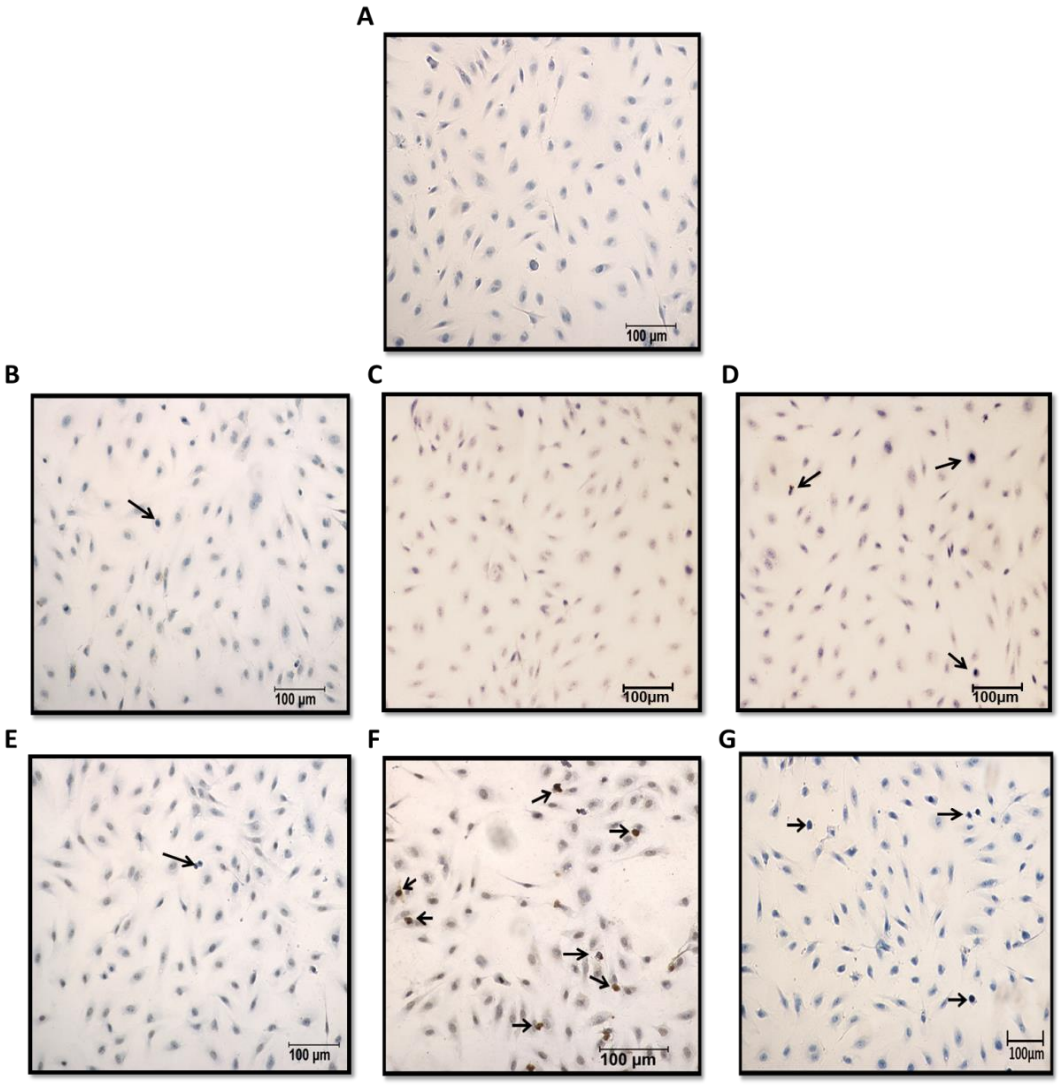


Figure 4.4: The effect of IV iron preparations on apoptosis in HUVEC evaluated by TUNEL Assay. TUNEL staining performed after HUVECs were pre-incubated with 50 µg/ml IV iron preparations for 2, 6 and 24hr. Black arrow indicates apoptotic cells (400X). **(A):** untreated HUVEC, **(B):** HUVEC treated with IV iron sucrose 2hr, **(C):** HUVEC treated with IV FCM 2hr, **(D):** HUVEC treated with IV iron sucrose 6hr, **(E):** HUVEC treated with IV FCM 6hr, **(F):** HUVEC treated with IV iron sucrose 24hr and **(G):** HUVEC treated with IV FCM 24hr.

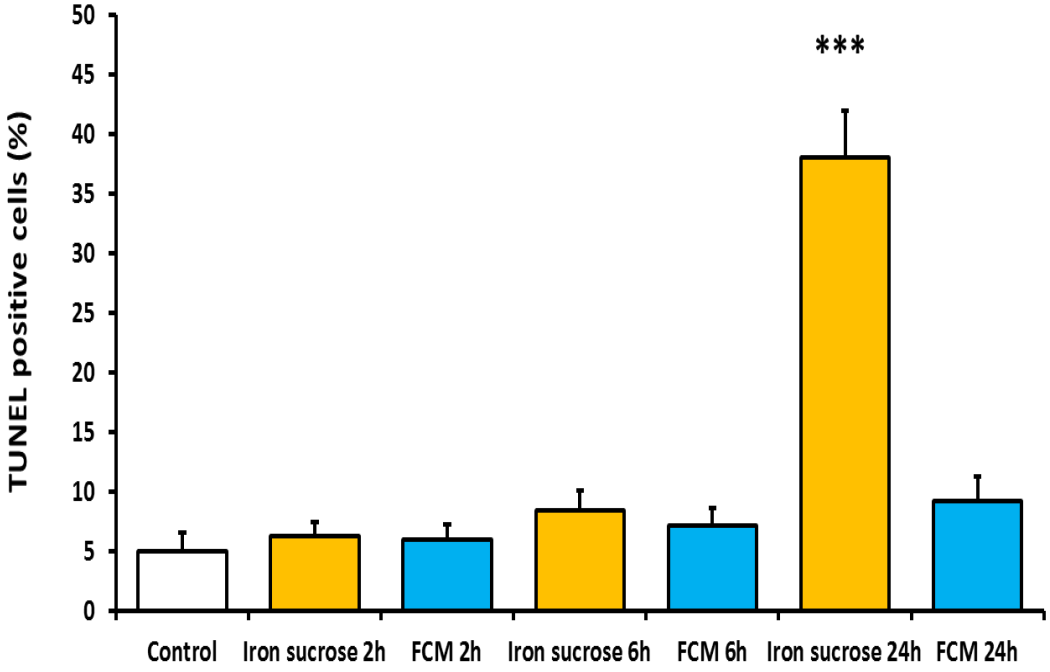
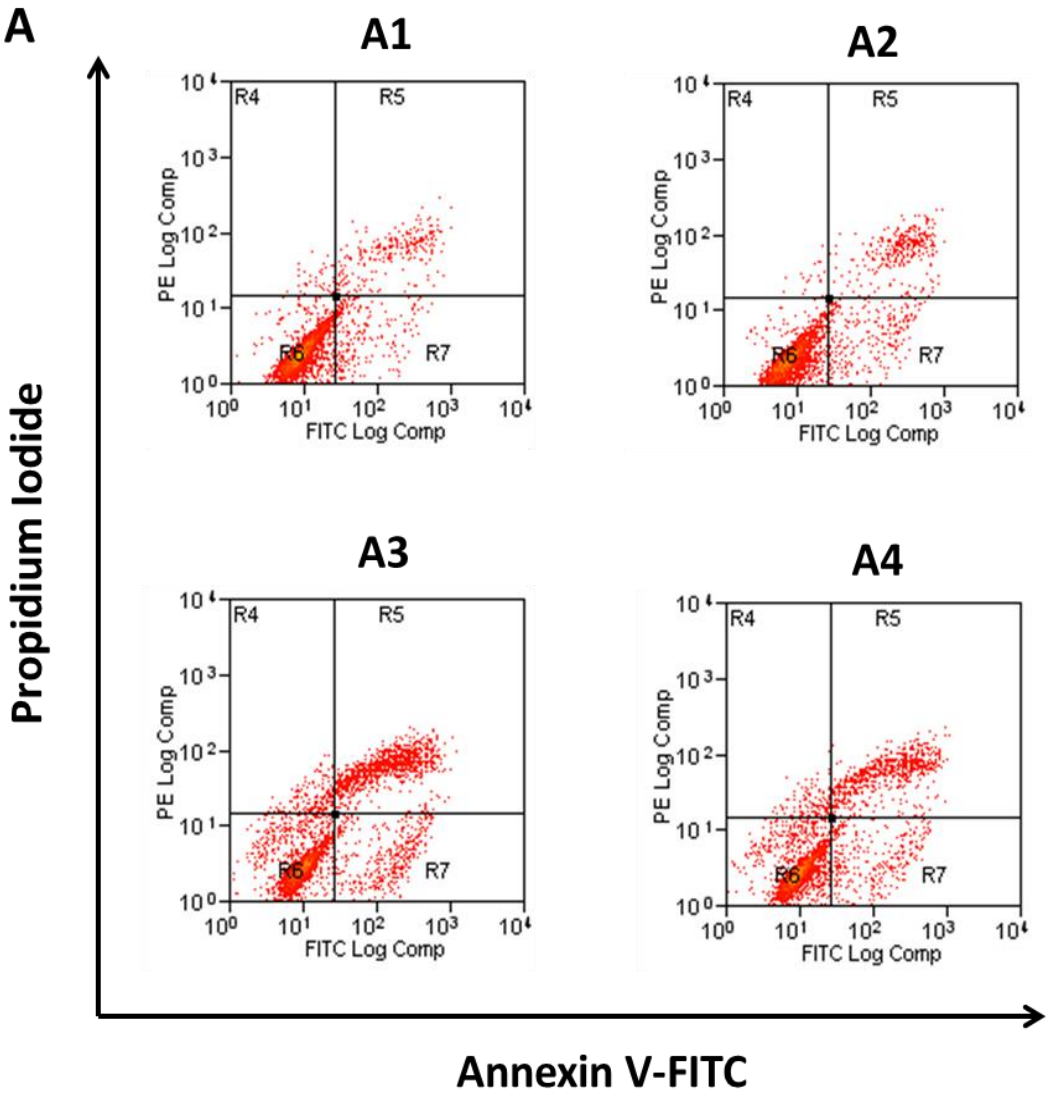


Figure 4.5: The effect of IV iron preparation on percentage of HUVEC apoptosis quantified by TUNEL assay. Apoptotic cells that show irregular nuclear staining were counted in four different random image fields, each containing approximately 100 cells from each culture. Degrees of apoptotic cell death are expressed as percentages compared to total cell number within each field. Values are expressed as (mean ± SD) from three different donors (n=3). ****p*<0.001 for IV iron sucrose at 24hr compared with non-treated cells or cells treated with IV FCM at 24hr.

4.3.2. Effects of iron preparations on the apoptosis in HUVEC using Annexin V-FITC/PI staining by flow cytometry

The percentage of early IV iron-treated HUVEC apoptosis was also confirmed using the Annexin V-FITC/PI Apoptosis Detection kit (Abcam). Stained cells were analysed by flow cytometry (FACScaliber; Beckman Coulter, Inc. UK). Viable cells, were those staining negative for Annexin V and PI, early apoptotic cells were positive for Annexin V and negative for PI staining while late apoptotic cells were Annexin V and PI staining positive (Figure 4.6A). Annexin V-FITC/PI showed that HUVEC treated with IV FCM at 24hr were not significantly different to non-treated cells (Figure 4.6B). On the other hand, cells treated with IV iron sucrose at 24hr showed significant increase in the apoptosis of HUVEC compared with IV FCM-treated HUVEC or non-treated cells (p<0.001) (Figure 4.6, A6) The percentage of apoptosis was 5.19 ± 0.53% in non-treated cells, 7.12 ± 0.89% in IV FCM, significantly increased to 35.18 ± 3% in iron sucrose-treated HUVEC (Figure 4.6B). This increase was significantly attenuated by p38 MAPK inhibitor SB203580 compared with cells treated with IV iron sucrose alone (p<0.05) (Figure 4.6B). However, the data was still significant compared with non-treated cells (p<0.05) (Figure 4.6B), and the percentage of apoptosis was 20.10 ± 0.53% in IV iron sucrose and SB203580 treated cells.


A
A1
A2
A3
A4
R4
R5
R6
R7
PE Log Comp
FITC Log Comp
Propidium Iodide
Annexin V-FITC

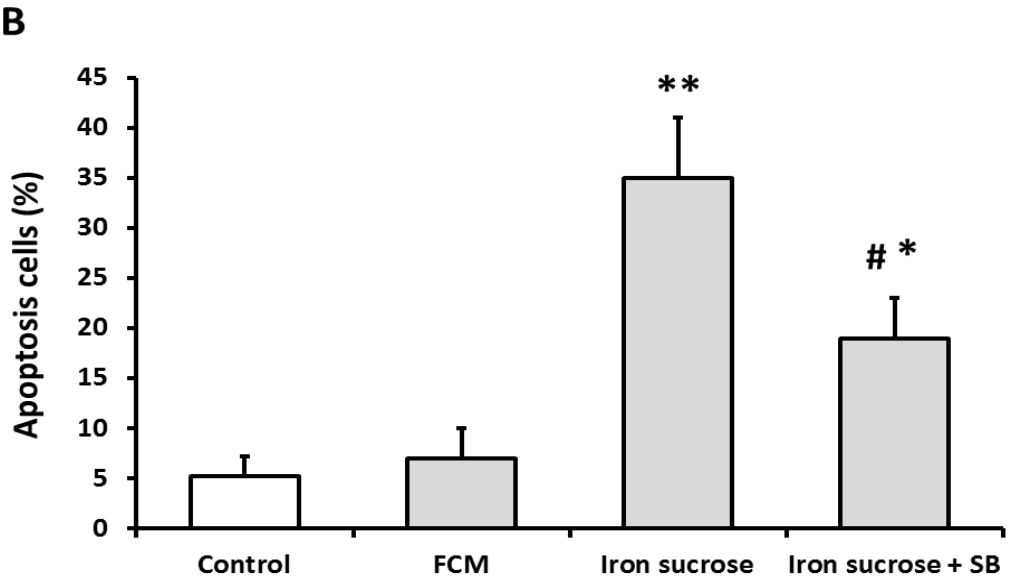


Figure 4.6: The effect of IV iron preparations on apoptosis of HUVEC using Annexin V-FITC/PI by flow cytometry (A-B). HUVEC were treated with 50 µg/ml IV iron sucrose or IV FCM for 24hr or left untreated as control. In addition cells were treated with SB203580 and IV iron sucrose for 24hr to examine the effect of p38 MAPK on apoptosis induced by iron sucrose, and then incubated at 37^0C in 5% CO_2. Subsequently, HUVEC were harvested and labelled with a combination of Annexin V-FITC and PI for 15 min in dark. **(A: A1-A4):** Flow cytometry dot plot figures of apoptotic cells, (A1): Non-treated cells (control) group, (A2): IV FCM group, (A3): IV iron sucrose group, (A4): IV iron sucrose and SB203580. In each dot plot figure, the upper left quadrant corresponds to necrotic cells, which are negative for Annexin and PI positive (R4); the upper right quadrant contains the later apoptotic cells, which are positive Annexin V positive and PI positive cells (R5); the lower left quadrant shows viable cells, Annexin V negative and PI negative (R6) and the lower left quadrant represents the early apoptotic cells, Annexin V positive and PI negative (R7). **(B):** shows a column bar graph to quantify flow cytometry analysis of the percentage of apoptotic cells. All data are expressed as (means ± SD) from three different donors (n=3). ** p<0.01 for IV iron sucrose compared to non-treated cells (control), * p<0.05 for IV iron sucrose and SB203580 versus non-treated cells and $^{\#}p$<0.05 for IV iron sucrose and SB203580 at 24hr versus IV iron sucrose at 24hr alone.

4.3.3. Western Blot analysis

4.3.3.1. The effects of IV iron preparations on phosphorylation of p38 MAPK and expression of Bcl-2 and Bax

4.3.3.1.1. The effects of IV iron preparations on the phosphorylation of p38 MAPK

HUVEC were cultured in complete endothelial growth medium containing 50 µg/ml of IV iron sucrose or IV FCM for 15 min, 30 min, 60 min, 2, 6 and 24hr. Antibodies against phosphorylated p38 MAPK were used to detect p38 MAPK phosphorylation in the cell lysates. Results from repeated protein assays showed the protein concentration in the cell lysates from confluent 6 well plates as shown below 0.9 mg/ml (Figure 4.6).

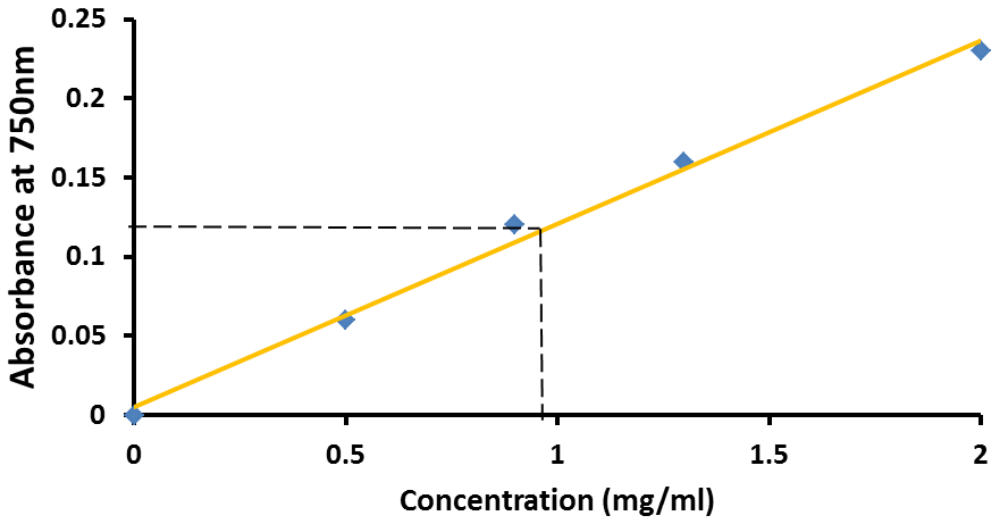


Figure 4.7: Graph of Protein assay standard curve shows the absorbencies and concentrations of the standards. A serial of dilution of BSA in PBS was prepared and absorbance was read at 750 nm. The absorbance of this sample was 0.13 and when it is plotted on the graph, protein concentration showed to be 0.9 mg/ml.

To determine whether the stimulation of HUVEC by IV iron preparations sucrose or IV FCM resulted in detectable phosphorylated p38 MAPK proteins, Western blotting was conducted.

Figures 4.8A and 4.9A show a protein band of 43 kDa representing phosphorylated p38 MAP kinase for IV iron sucrose or IV FCM at a concentration of 50 µg/ml for increasing t times (15 min, 30 min, 60 min, 2, 6 and 24hr). All figures are representative of 3 different experiments from three different donors. The band densities were quantified by the ratios of phosphorylated p38 to total p38 MAP kinase expression in the cells using Image J and the results are shown as the mean ratio from 3 different experiments (mean +/- SD) (Figures 4.8B and 4.9B)

HUVEC grown in complete endothelial growth media, were considered as a negative control and showed a less intense band compared with IV iron sucrose or IV FCM-treated HUVEC, These results showed low phosphorylation of p38 MAP kinase in non-treated cells (Figures 4.8A and 4.9A). However, cells grown in endothelial growth media containing 50 µg/ml IV iron sucrose or IV FCM at 15 min-6hr showed obvious increased phosphorylation of p38 MAP kinase compared with non-treated cells (Figures 4.8A and 4.9A). HUVEC grown in media containing 50 µg/ml IV iron sucrose or IV FCM for 15 and 30 min showed a slight increase in phosphorylation of p38 MAP kinase compared with non-treated cells (Figures 4.8A and 4.9A). However, this increase was not statistically significant (Figures 4.7B and 4.9B), while IV iron sucrose at 60 min and 2hr showed a statistically significant increase in p38 MAPK activation versus non-treated cells ($p<0.01$) (Figure 4.8B). In addition, p38 MAPK was significantly activated in cells

treated with IV FCM for 60 min and 2hr compared with non-treated cells (p<0.05) (Figure 4.8B). The p38 MAPK showed to be activated over 6hr, but was not significant compared with non-treated cells and slowly declined (Figures 4.10B and 4.11B). Chapter 3 clearly showed that, IV iron sucrose or IV FCM induced ROS generation in HUVEC. Thus, in order, to investigate whether the phosphorylation of p38 MAPK by IV iron sucrose or IV FCM was ROS dependent. HUVEC were pre-treated with ROS scavenger, NAC (2 mM) and then treated with IV iron sucrose or IV FCM for different time course as described in section (4.2.2.1.6.7). The results showed that in HUVEC treated with NAC and IV iron sucrose or IV FCM at 60 min and 2hr, p38 MAPK phosphorylation was significantly reduced compared with HUVEC treated with sucrose or IV FCM alone at 60 min and 2hr (p<0.05) (Figure 4.10B and 4.11B). P-p38 and total p38 MAPK exposure time under UV using Chemidoc machine was kept constant between experiments.

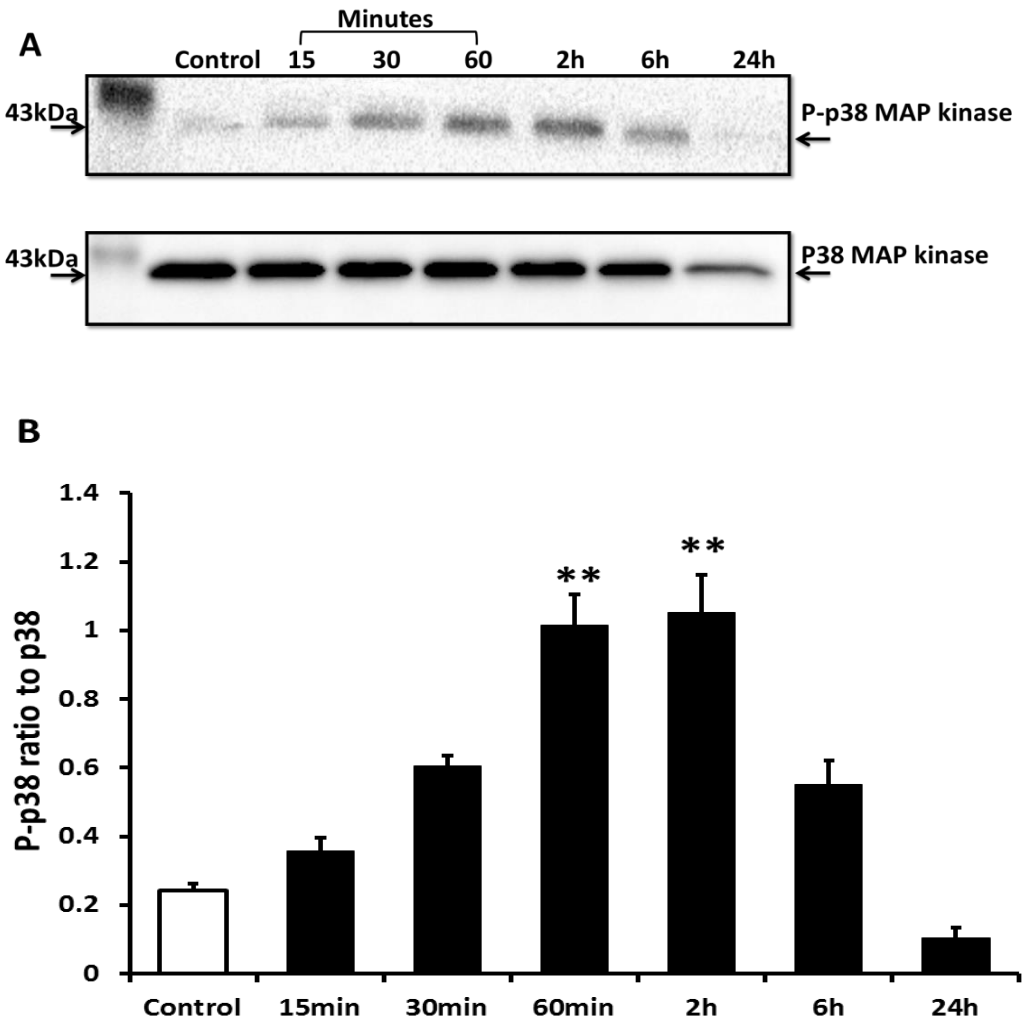


Figure 4.8: Shows the effect of IV iron sucrose on activation of p38 MAPK in HUVECs as determined by p38 phosphorylation. HUVECs were cultured in 6 well plates until confluence, then cells were cultured in the absence or presence of 50 µg/ml IV iron sucrose for 15, 30, 60 min, 2hr, 6hr and 24hr and lysed. 30-40 µg protein from each sample was loaded on an SDS-PAGE gel and used in the Western blot. **(A):** representative blots; the phospho-p38 and total p38 MAPK proteins were detected using specific antibodies and were identified by Western blot as a band of approximately 43 kDa. **(B):** the phospho-p38/total p38 ratio were quantified by densitometry and expressed as the ratio, compared to the control. All values are expressed as mean (+/- SD) from three different experiments (n=3). ** $p<0.01$ compared with non-treated cells.

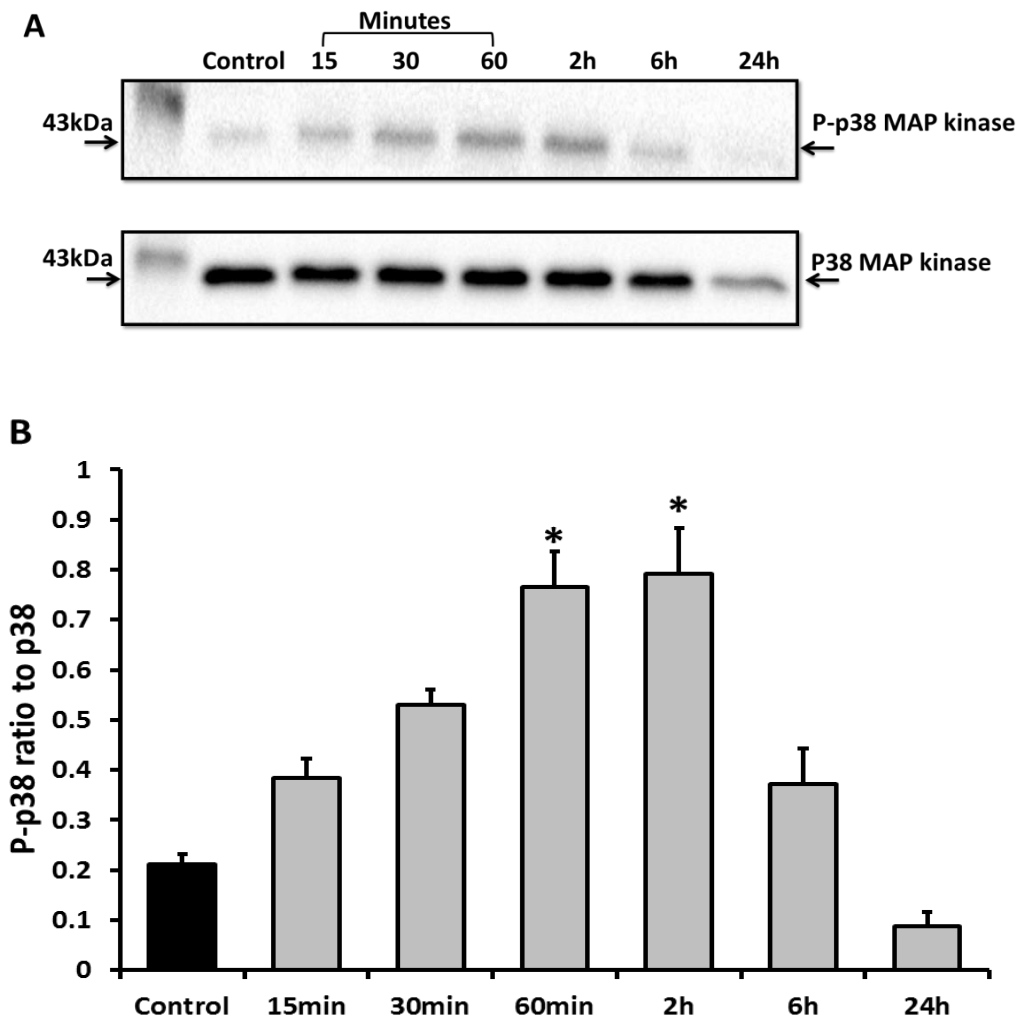


Figure 4.9: Shows the effect of IV FCM on activation of p38 MAPK in HUVECs. HUVECs were cultured in 6 well plates until confluence, then cells were treated in the absence or presence of 50 µg/ml IV FCM for 15, 30, 60 min, 2hr, 6hr and 24hr and lysed. 30-40 µg protein from each sample was loaded on an SDS-PAGE gel and used in the Western blot. **(A):** representative blots; the phospho-p38 and total p38 MAPK proteins were detected using specific antibodies and were identified by Western blot as a band of approximately 43 kDa. **(B):** the phospho-p38/total p38 ratio were quantified by densitometry and expressed as the ratio, compared to the control. All values are expressed as mean (+/- SD) from three different experiments (n=3). *p<0.05 compared with non-treated cells.

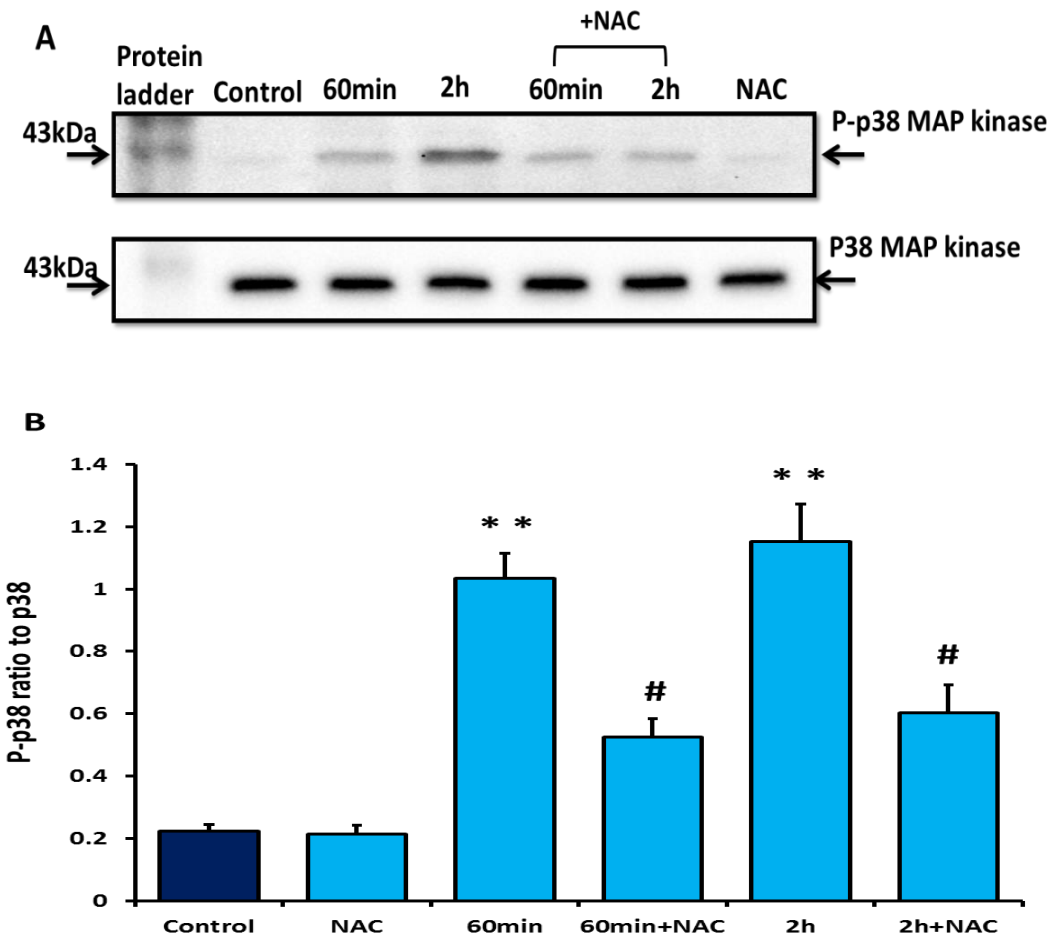


Figure 4.10: Shows the effects of NAC and IV iron sucrose on p38 MAPK phosphorylation in HUVECs. HUVECs were cultured in 6 well plates until confluence, then cells were incubated in the absence or presence of 2 mM NAC and 50 µg/ml IV iron sucrose for 60 min and 2hr, and lysed. 30-40 µg protein from each sample was loaded on an SDS-PAGE gel and used in the Western blot. **(A):** representative blots; the phospho-p38 and total p38 MAPK proteins were detected using specific antibodies and were identified by Western blot as a band of approximately 43 kDa. **(B):** the phospho-p38/total p38 ratio were quantified by densitometry and expressed as the ratio compared to the control. All values are expressed as mean (+/- SD) from three different experiments (n=3). **p<0.01 versus non-treated cells and $^{\#}p$<0.05 for IV iron sucrose and NAC at 60 min and 2hr compared with IV iron sucrose alone at 60 min and 2hr.

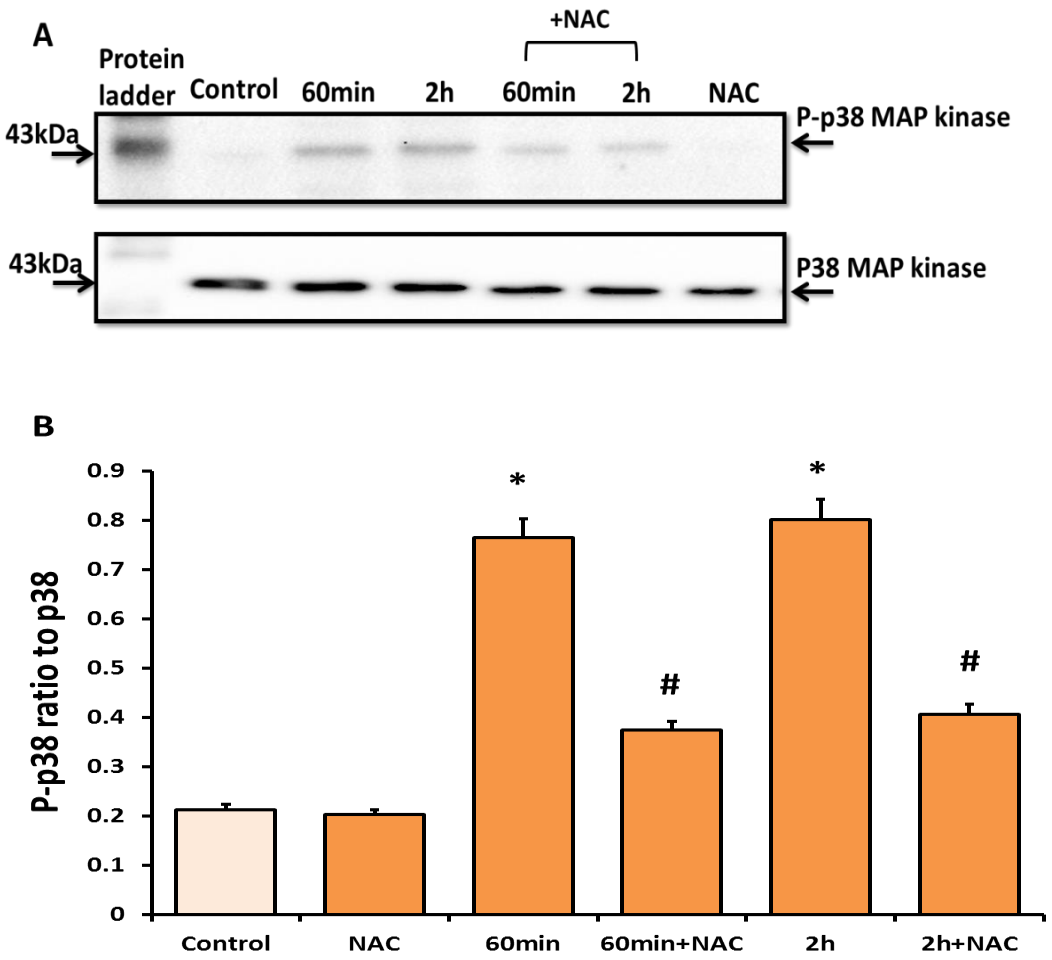


Figure 4.11: Shows the effects of NAC and IV FCM on p38 MAPK phosphorylation in HUVECs. HUVECs were cultured in 6 well plates until confluence, and then cells were incubated in the absence or presence of 2 mM NAC and 50 µg/ml of IV FCM for 60 min and 2hr, and lysed. 30-40 µg protein from each sample was loaded on an SDS-PAGE gel and used in the Western blot. **(A):** representative blots; the phospho-p38 and total p38 MAPK proteins were detected using specific antibodies and were identified by Western blot as a band of approximately 43 kDa. **(B):** the phospho-p38/total p38 ratio were quantified by densitometry and expressed as the ratio compared to the control. All values are expressed as mean (+/-SD) from three different experiments (n=3). *p<0.05 versus non-treated cells and $^{\#}p$<0.05 for IV FCM and NAC at 60 min and 2hr compared with IV FCM alone at 60 min and 2hr .

4.3.3.1.2. The effect of IV iron preparations on expression of Bcl-2 and Bax proteins by Western blot

Bcl-2 protein family members, such as Bcl-2 have been shown to play an important role in the regulation of apoptosis by blocking cytochrome *c* release from the mitochondria (Gogvadze *et al*., 2006). In order, to investigate the role of IV iron sucrose or IV FCM on mitochondria membrane integrity through apoptosis stress pathway protein expression, Western blots were conducted using antibodies against Bcl-2 and Bax. The results showed that Bcl-2 protein expression was not changed or decreased after HUVEC were treated with 50 µg/ml IV FCM for 2, 6 and 24hr or 50 µg/ml IV iron sucrose at 2 and 6hr compared with non-treated cells (Figures 4.12A and 4.14A). However, Bcl-2 protein expression was significantly down-regulated in HUVEC treated with IV iron sucrose at 24hr compared with non-treated cells ($p<0.05$) (Figure 4.12B). In addition, protein expression of Bax was not up-regulated in HUVEC treated with IV FCM at 2, 6 and 24hr compared with non-treated HUVEC (Figure 4.15B). In contrast, HUVEC treated with IV iron sucrose at 24hr showed a significant up regulation in Bax protein expression compared with non-treated cells ($p<0.05$) (Figure 4.13B). Several studies showed that SB203580 play a key role in inhibiting or reducing the activity of p38 MAPK signalling pathway, and therefore prevent cell death in different cell types include ECs such as EA.hy926 and pulmonary microvascular ECs (PMVECs) (Grethe *et al*., 2004; Yang *et al*., 2012; Liu *et al*., 2014). Thus, in order to examine potential links between the p38 MAPK signalling pathway and apoptosis in HUVEC, the effect of SB203580 on the expression of Bcl-2 and Bax in HUVEC treated with IV iron sucrose was examined. HUVEC were

pre-treated with 10 μM SB203580 for 1hr and then treated with IV iron sucrose for 24hr, and Western blotting method was performed as described in section (4.2.2.1.6.8). HUVEC treated with both IV iron sucrose and SB203580 for 24hr showed both a significant increase in Bcl-2 protein expression compared with cells treated with IV iron sucrose alone at 24hr (p<0.05) (Figure 4.16B), and a significant decrease in Bax expression (p<0.05) (Figure 4.17B). These results suggest that exposure to IV iron sucrose causes EC apoptosis via activation of p38 MAPK. In contrast, IV FCM did not induce apoptosis or alter Bcl-2/Bax expression. Furthermore, SB203580 showed to have a protective role against IV iron sucrose-induced apoptosis in HUVEC by regulating the expression of apoptosis-related proteins Bcl-2 and Bax. Our results for SB203580 inhibitor and IV iron compounds can be criticised as control was not treated with 0.1% DMSO alone and in this case it can not be used as appropriate control. However, previous work in our lab and other published studies showed that this final concentration of solvent has no effect on HUVEC or ROS generation (Alkhoury, 2009; Li *et al*., 2010; Omae *et al*., 2013).

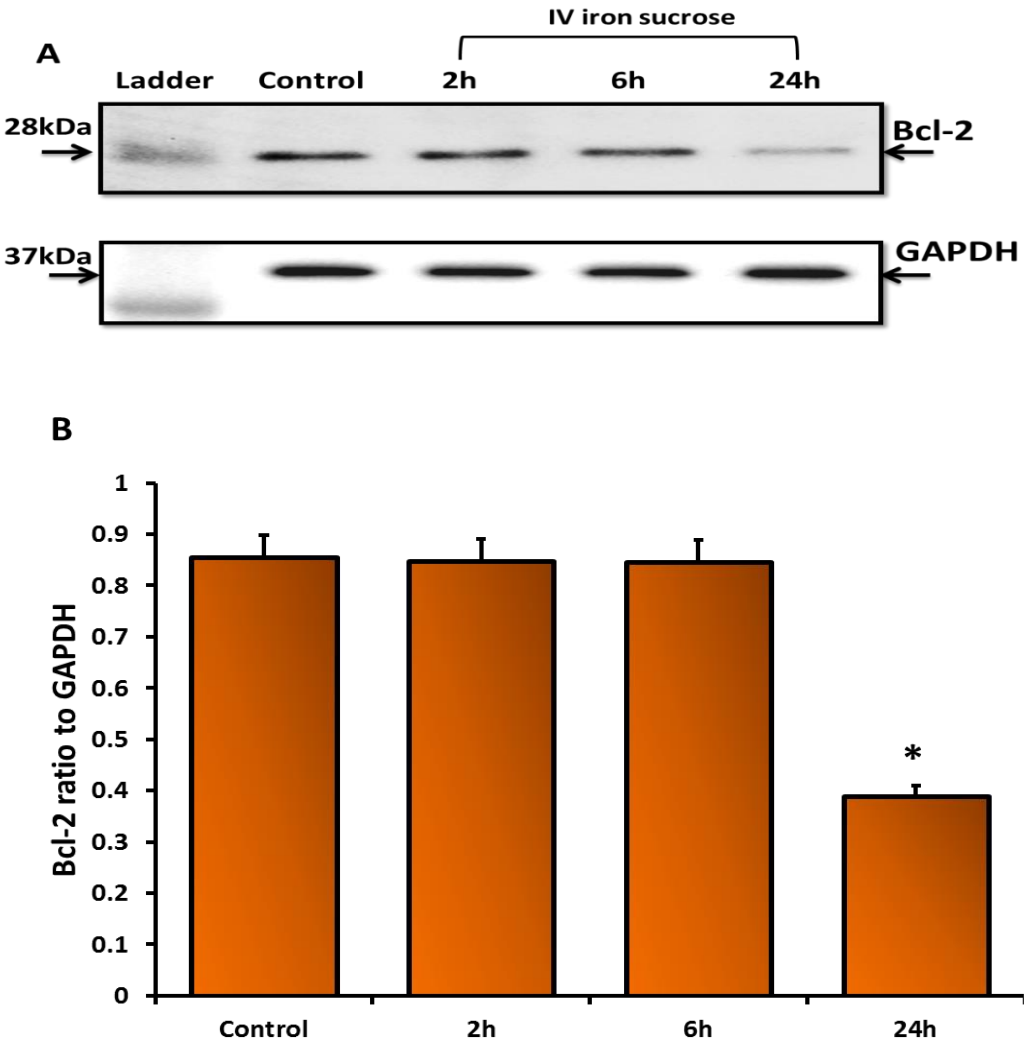


Figure 4.12: Western blot analysis of Bcl-2 protein expression in HUVEC treated with IV iron sucrose. Cells were treated with 50 µg/ml for 2, 6 and 24hr, cells were then lysed, and 30 µg protein extracts were subjected to Western blot analysis using specific antibodies against Bcl-2. **(A):** representative blots; the Bcl-2 and GAPDH proteins were detected by Western blot as a band of approximately 28 kDa and 37 kDa respectively. **(B):** the Bcl-2/GAPDH ratio were quantified by densitometry and expressed as the ratio to the control. All values are expressed as mean (+/- SD) from three different experiments (n=3). *p<0.05 for IV iron sucrose at 24hr compared with non-treated cells (control).

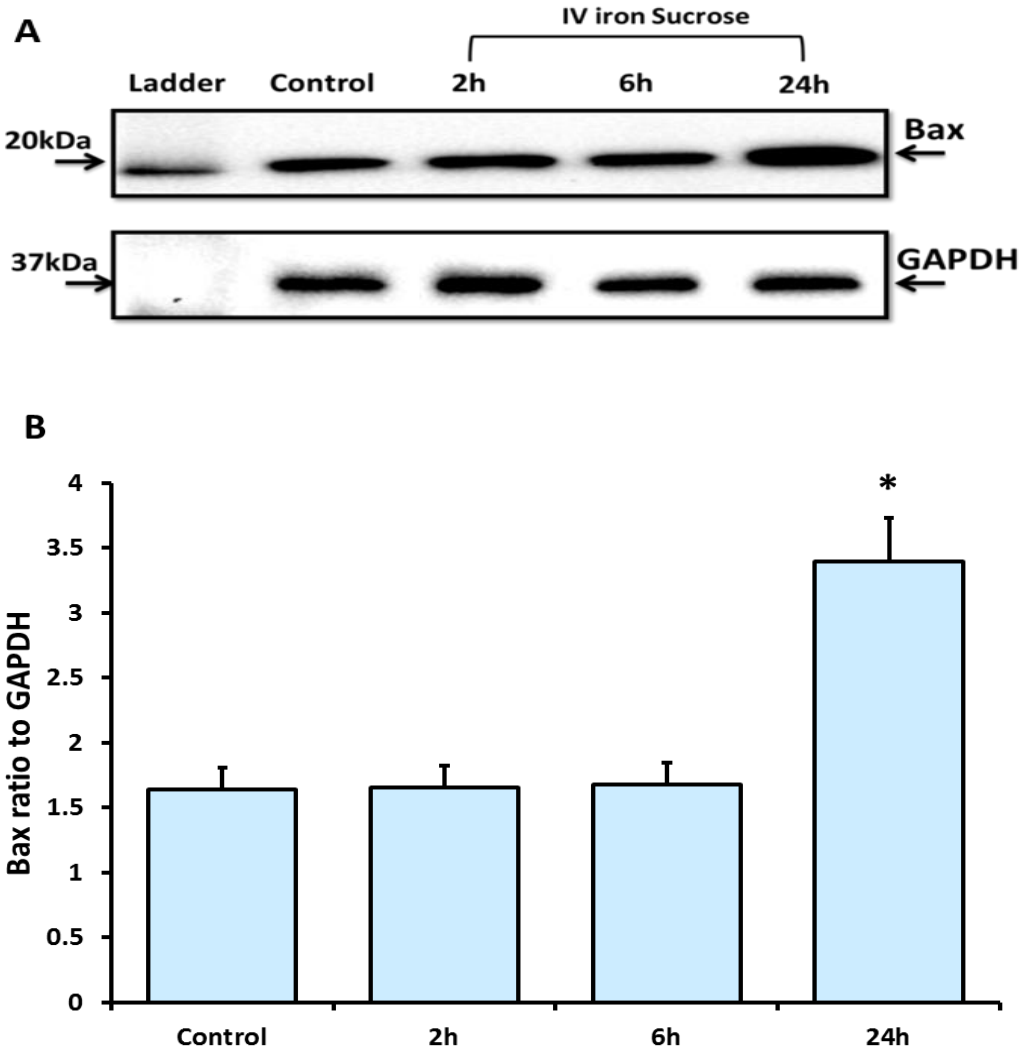


Figure 4.13: Western blot analysis of Bax protein expression in HUVEC by IV iron sucrose. Cells were treated with 50 µg/ml for 2, 6 and 24hr, cells were then lysed, and 30 µg protein extracts were subjected to Western blot analysis using specific antibodies against Bax. **(A):** representative blots; the Bax and GAPDH proteins were detected by Western blot as a band of approximately 20 kDa and 37 kDa respectively. **(B):** the Bax/GAPDH ratio were quantified by densitometry and expressed as the ratio to the control. Result from one experiment of three (n=3) performed is shown. All data are expressed as (mean ± SEM). *p<0.05 for IV iron sucrose at 24hr compared with non-treated cell (control).

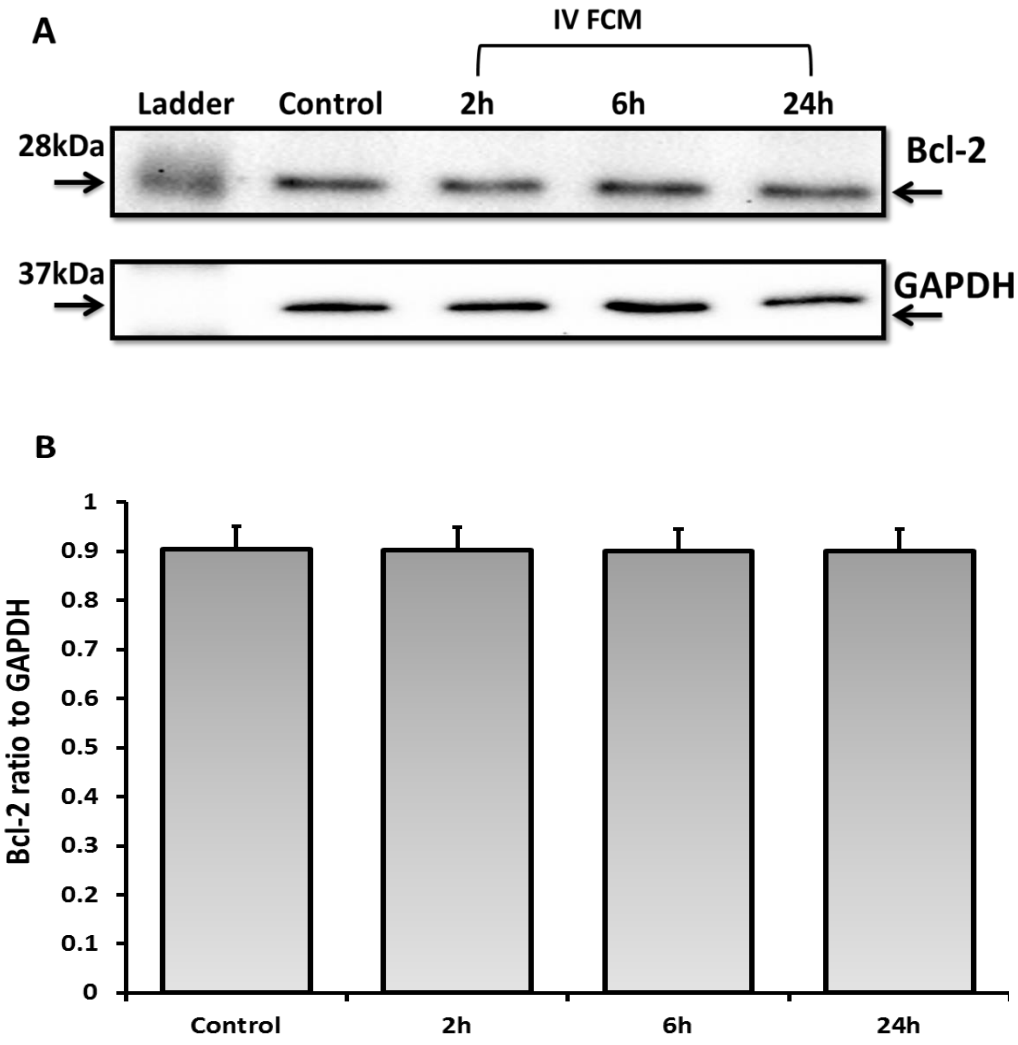


Figure 4.14: Western blot analysis of Bcl-2 protein expression in HUVEC by IV FCM. Cells were treated with 50 µg/ml for 2, 6 and 24hr, cells were then lysed, and 30 µg protein extracts were subjected to Western blot analysis using specific antibodies against Bax. **(A):** representative blots; the Bcl-2 and GAPDH proteins were detected by Western blot as a band of approximately 28 kDa and 37 kDa respectively. **(B):** the Bax/GAPDH ratio were quantified by densitometry and expressed as the ratio to the control. All values are expressed as mean (+/- SD) from three different experiments (n=3).

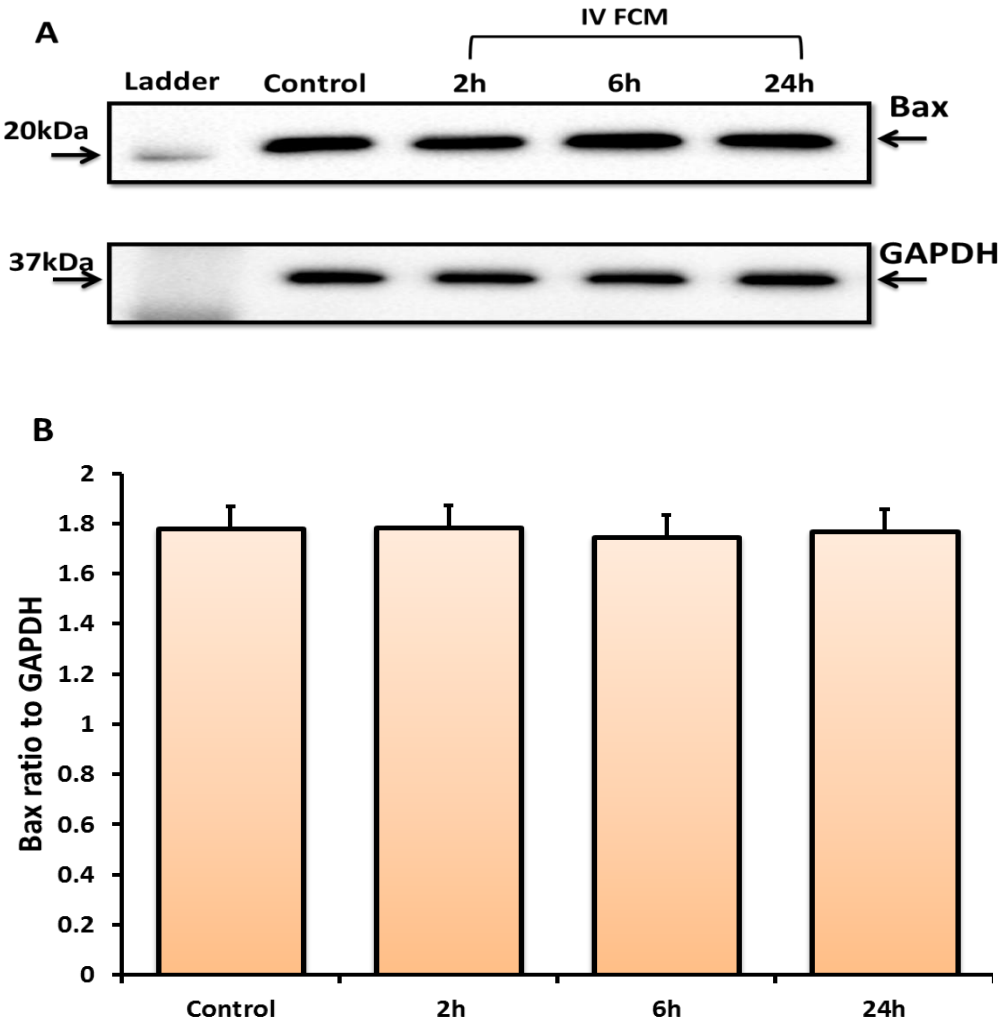


Figure 4.15: Western blot analysis of Bax protein expression in HUVEC by IV FCM. Cells were treated with 50 µg/ml for 2, 6 and 24hr, cells were then lysed, and 30 µg protein extracts were subjected to Western blot analysis using specific antibodies against Bax. **(A):** representative blots; the Bax and GAPDH proteins were detected by Western blot as a band of approximately 20 kDa and 37 kDa respectively. **(B):** the Bax/GAPDH ratio were quantified by densitometry and expressed as the ratio to the control. All values are expressed as mean (+/- SD) from three different experiments (n=3).

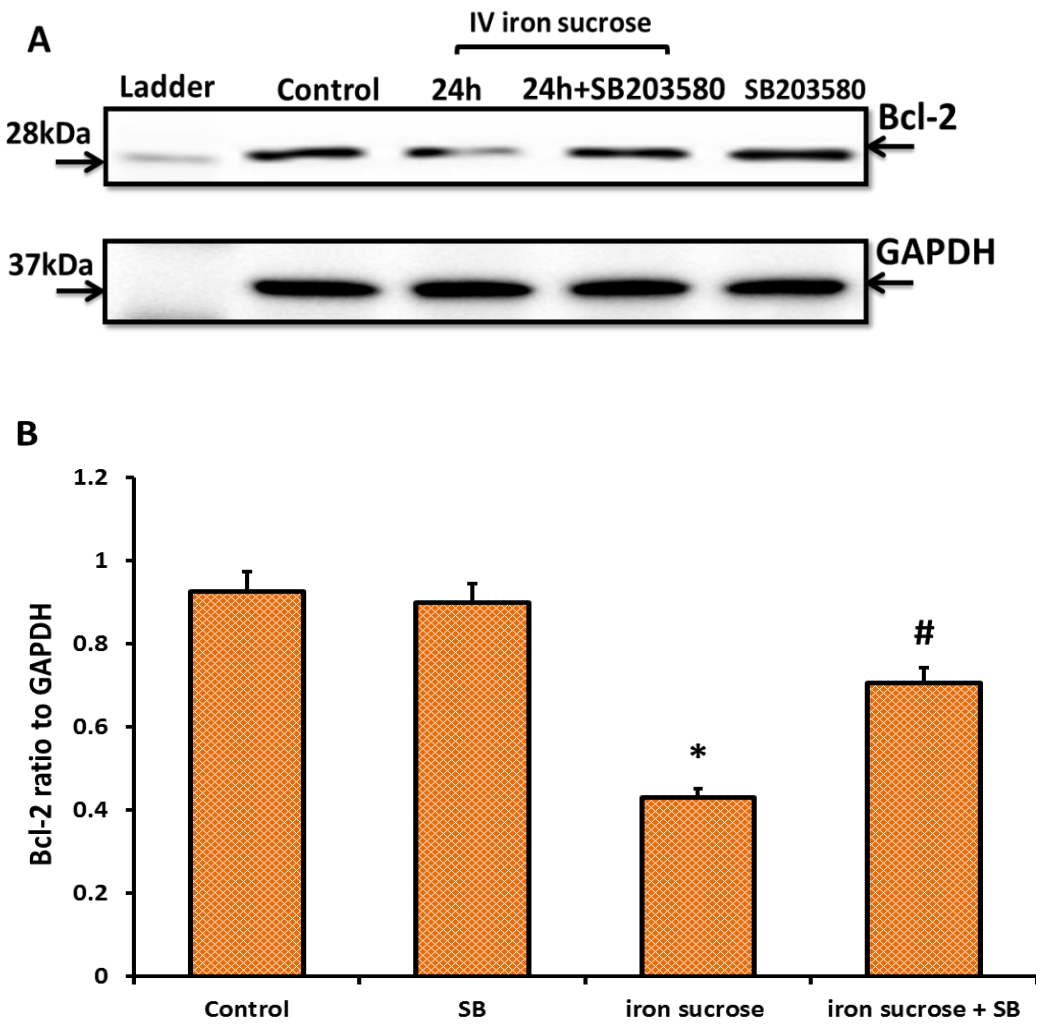


Figure 4.16: The effect of IV iron sucrose and p38 MAPK inhibitor on Bcl-2 expression in HUVEC. HUVEC were pre-treated with 10 µM SB203580 for 60 min, and then stimulated with 50 µg/ml IV iron sucrose for 24hr. **(A):** representative blots; the Bcl-2 and GAPDH proteins were detected by Western blot as bands of approximately 28 kDa and 37 kDa respectively. **(B):** the Bcl-2/GAPDH ratio were quantified by densitometry and expressed as the ratio to the non-treated cells (control). All values are expressed as mean (+/- SD) from three different experiments (n=3). *p<0.05 compared with non-treated cells and $^{\#}p$<0.05 for IV iron sucrose and SB203580 at 24hr compared with IV iron sucrose at 24hr alone.

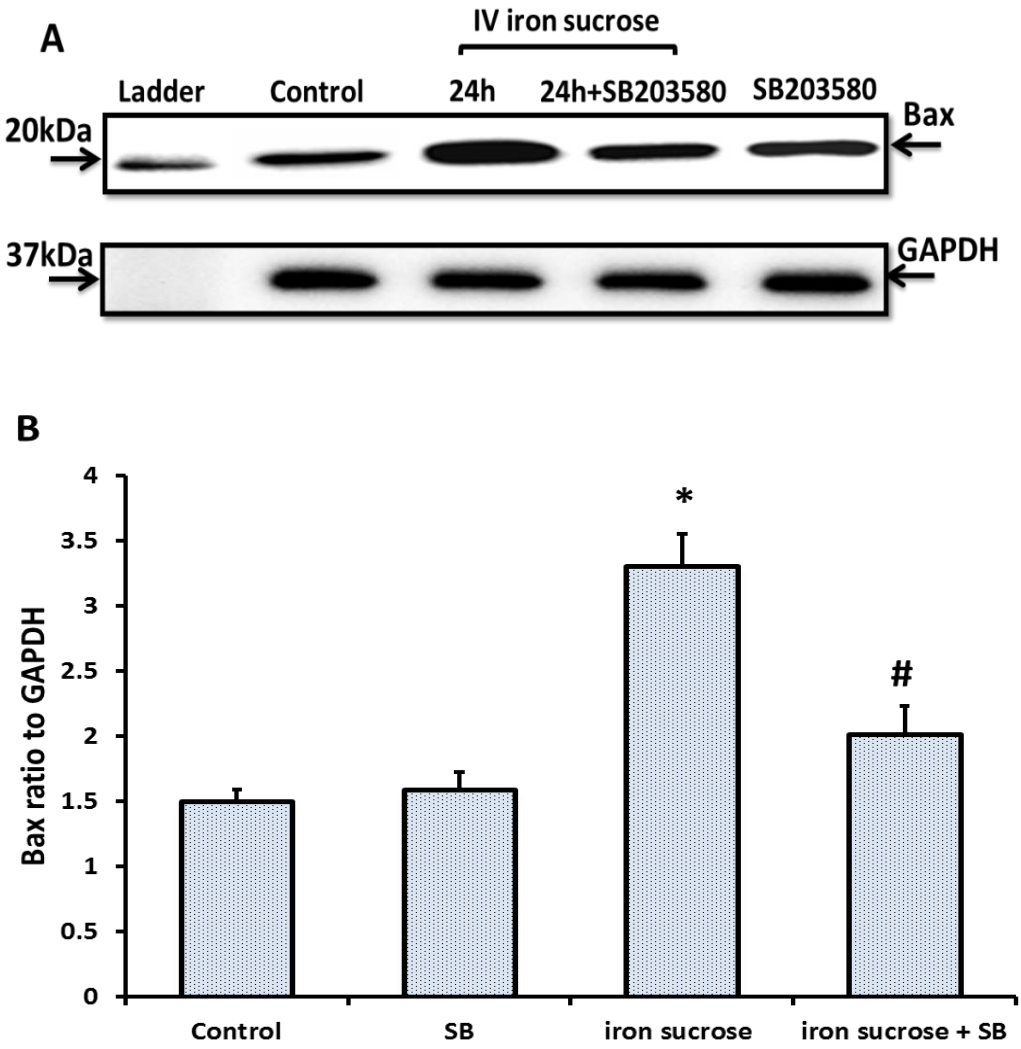


Figure 4.17: The effect of IV iron sucrose and p38 MAPK inhibitor on Bax expression in HUVEC. HUVEC were pre-treated with 10 µM SB203580 for 60 min, and then stimulated with 50 µg/ml IV iron sucrose for 24hr. **(A):** representative blots; the Bax and GAPDH proteins were detected by Western blot as bands of approximately 20 kDa and 37 kDa respectively. **(B):** the Bax/GAPDH ratio were quantified by densitometry and expressed as the ratio to the control. All values are expressed as mean (+/- SD) from three different experiments (n=3). **p*<0.05 compared with non-treated cells and $^{\#}p$<0.05 for IV iron sucrose and SB203580 at 24hr compared with IV iron sucrose at 24hr alone.

Table 4.1: Summary table of Western blot data presenting an overall reference point for p38 MAPK, Bcl-2 and Bax expression changes induced by IV iron compounds. Data shown represent group values are (mean ± SEM) of three experiments (n=3). *p <0.05, **p <0.01 and ***p<0.001 versus control and (ns) = not significant.

IV iron compound	**Proteins of interest and/or treatment**		
IV iron sucrose	**P38 MAPK**	**IV iron sucrose and NAC**	**P38 MAPK**
Control	0.24± 0.06	**Control**	0.22±0.04
15 min	0.35 ± 0.04 ns	**NAC**	0.21±0.03
30min	0.60± 0.06 ns	**60 min**	1.03±0.10**
60 min	1.01± 0.10**	**60 min+NAC**	0.52±0.06#
2 hr	1.05±0.14**	**2 hr**	1.07±0.15**
6 hr	0.55±0.04 ns	**2 hr+NAC**	0.60±0.08#
24 hr	0.10±0.02 ns		
IV FCM	**P38 MAPK**	**IV FCM and NAC**	**P38 MAPK**
Control	0.21± 0.02	**Control**	0.23±0.02
15 min	0.38± 0.04 ns	**NAC**	0.22±0.03
30min	0.53±0.05 ns	**60 min**	0.77±0.08*
60 min	0.76± 0.09*	**60 min+NAC**	0.39±0.1#
2 hr	0.79±0.11*	**2 hr**	0.80±0.15*
6 hr	0.37±0.03 ns	**2 hr+NAC**	0.40±0.06#
24 hr	0.08± 0.01 ns		
IV iron sucrose	**Bcl-2**	**Bax**	
Control	0.85±0.04	1.64±0.25	
2 hr	0.84±0.05ns	1.65±0.23ns	
6 hr	0.84±0.03ns	1.66±0.26ns	
24 hr	0.39±0.02*	3.3±0.32*	
IV FCM	**Bcl-2**	**Bax**	
Control	0.92±0.03	1.77±0.20	
2 hr	0.91±0.04ns	1.78±0.22ns	
6 hr	0.92±0.03ns	1.74±0.25ns	
24 hr	0.90±0.02ns	1.76±0.22ns	
IV iron sucrose and SB203580 at 24hr	**Bcl-2**	**Bax**	
Control	0.87±0.06	1.49±0.24	
SB203580 alone	0.89±0.04	1.58±0.26	
IV iron sucrose alone	0.43±0.03*	3.30±0.34*	
IV iron sucrose + SB203580	0.70±0.05#	2.01±0.28#	

4.4. DISCUSSION

Apoptotic pathways are an important mechanism resulting in programmed cell death by altering mitochondrial membrane potential (Elmore *et al*., 2007). Most importantly, apoptosis of ECs can critically disturb the integrity of the endothelial monolayer, which contributes to vascular damage and is an early step in atherogenesis (Haimovitz-Friedman *et al*., 1997). It has certainly been shown that, in CKD patients, acceleration of atherosclerosis can be as a result of endothelial injury (Ross, 1995; Malyszko, 2010). IV iron preparations which are frequently used for the management of anaemia in CKD patients, promote apoptosis in cultured endothelial HAECs (Kamanna *et al*., 2012) but the mechanisms responsible are unclear. Therefore, the role of IV iron sucrose and IV FCM were examined on our model HUVEC to determine cell activation and damage. The present study demonstrates that IV iron sucrose causes significant EC apoptosis. This effect was demonstrated using both TUNEL assay and with Annexin V-FITC/PI using flow cytometry following 24hr treatment with IV iron sucrose compared to non-treated cells (Figure 4.5). TUNEL assay is well known as a standard method for late apoptosis detection in both cell culture and within tissue (Darzynkiewicz *et al*., 2008). However, since DNA damage can occur as a feature of both apoptosis and necrosis, the accuracy of TUNEL assay results as a measure of apoptosis has been questioned in several studies (Grasl-Kraupp *et al*., 1995; Orita *et al*., 1999). Therefore, to confirm our results reflected EC apoptosis in HUVEC, apoptosis was also investigated by Annexin V-FITC/PI. The data showed that, apoptosis of HUVEC treated with IV FCM slightly increased compared to non-treated cells and this increase was not significant

compared with non-treated cells (Figure 4.6B). In contrast, IV iron sucrose showed significant increase in apoptosis of HUVEC compared with non-treated cells (Figure 4.6B). These results confirmed the data obtained from TUNEL assay method and showed that IV iron sucrose was the most injurious to HUVEC. These findings are in agreement with a study by Carlini and colleagues who showed that, treating BAECs with 100 and 1000 µg/ml IV iron sucrose for 24hr, significantly increased the apoptosis by 20.4 and 38.3% respectively (Carlini *et al*., 2006). Our data demonstrates that our HUVEC model is more sensitive to apoptosis than BAEC. These authors suggested that the effect of IV iron sucrose on BAEC death was due to mitochondrial dysfunction induced by this compound, which was confirmed by the decreased expression of Bcl-2.

Our data of apoptosis by Annexin V-FITC/PI are in line with the findings of Martin-Malo *et al* (2012), who used the same method and showed that treating PBMC from CKD patients at stage 5 on HD with 200 µg/ml IV iron sucrose or IV FCM for 8hr, resulted in a significant increase in apoptotic PBMC compared with non-treated cells ($p<0.05$). The authors suggested that PBMC apoptosis was significantly increased primarily due to the increased oxidative stress that is caused by HD, and subsequently worsened by the IV iron infusion. The role of HD in initial generation of oxidative stress, compounded with additional oxidative stress due to IV iron could explain why IV FCM had significantly induced the apoptosis of PBMC. While the use of healthy primary HUVEC and low concentration of 50 µg/ml of IV iron sucrose or IV FCM in our studies may explain the lack of effects of IV FCM on apoptosis and may be more relevant to clinical dose regimes. Interestingly,

this also shows that even a clinically relevant, relatively low concentration of IV iron sucrose is capable of inducing EC death. Similar findings have been described in another study, which was performed on blood samples from ten healthy individuals. Each blood sample was divided and IV iron sucrose was added to reach final concentrations of 20, 100 and 200 mg/L for 4 and 24hr, to investigate the effect of this compound on apoptosis of polymorph nuclear Leukocytes (PMN), using Annexin V-FITC/PI method. The authors found that apoptosis was significantly induced by IV iron sucrose at only 24hr, and suggested that this is most likely due to iron induced oxidative stress in these cells (Ichii *et al*., 2012). To investigate the mechanism of IV iron sucrose-mediated apoptosis, HUVEC were pre-treated with SB203580, then IV iron sucrose and at 24hr, flow cytometry analysis using Annexin V-FITC/PI was performed. This data, showed partly but significantly reduced apoptosis of HUVEC compared with cell treated with IV iron sucrose alone at 24hr (Figure 4.6B), confirming that activation of p38 MAPK is involved in HUVEC apoptosis induced by IV iron sucrose.

Bcl-2 proteins family, including Bcl-2 and Bax, are involved in the regulation of apoptosis (Soria *et al*., 2013). The balance of expression of these proteins plays a key role in apoptotic signal activation to the mitochondria and maintenance of cell morphology and function. Overexpression of Bcl-2 blocks the release of cytochrome *c* from the mitochondria to the cytosol, preventing caspase-3 activation and the induction of intrinsic pathway of cell apoptosis (Suen *et al*., 2008). Therefore, to confirm our data that IV FCM did not induce apoptosis of HUVEC and to investigate the mechanism involved in the HUVEC injury by IV iron sucrose, Bcl-2 and Bax expression following

IV iron preparation treatment was measured. The effect of IV iron sucrose on mitochondria integrity was associated with down-regulation of Bcl-2 and up-regulation of Bax protein expression, confirming the role of these two proteins in the apoptotic stress pathway. By Western blotting, IV FCM at 2, 6 and 24hr, or IV iron sucrose at 2 and 6hr did not down-regulate the protein expression of the anti-apoptotic Bcl-2 compared to non-treated HUVEC (Figures 4.12B and 4.14B). In addition, Bax protein expression also did not change in these cells compared with non-treated HUVEC (Figures 4.13B and 4.15B). In contrast, HUVEC treated with IV iron sucrose for 24hr showed significantly decreased Bcl-2 protein expression compared with non-treated cells (Figure 4.12B). In addition, expression of pro-apoptotic Bax was significantly increased in HUVEC treated with IV iron sucrose at 24hr compared with non-treated cells (Figure 4.13B). Since these two proteins are essential administrative components for caspase enactment and apoptosis, these changes in expression could lead to the altered mitochondrial permeability, resulting in the release of cytochrome *c* from the mitochondria to the cytosol, representing an irreversible step in cellular apoptosis. These results are in line with Carlini *et al*., (2006) who found that BAECs treated with 100 and 1000 µg/ml IV iron sucrose for 8-16hr, results in significant down-regulation of Bcl-2 protein expression compared with control (p<0.01). However, in their cells, even with higher concentrations of IV iron sucrose treatment, Bax protein expression did not change. The authors suggested that, the expression of Bax was not changed as both Bcl-2 and Bax might act independently. In contrast, our data showed that both proteins were affected

by IV iron sucrose, in a way which would promote apoptosis. However, for further discussion of these results see section 6.1.1.

As our study showed p38 MAPK activation to be involved in apoptosis of HUVEC in response to iron sucrose, p38 MAPK inhibition was used to determine whether the IV iron sucrose induced changes in Bcl-2 and Bax could be blocked. Several studies showed that p38 MAPK have a role in early induction of the apoptosis by mediating the phosphorylation of Bcl-2 and the translocation of Bax to the mitochondria (De Chiara *et al*., 2006; Kang *et al*., 2008; Park *et al*., 2013). Our study showed p38 MAPK was phosphorylated by 50 µg/ml IV iron sucrose or IV FCM at both 60 min and 2hr compared with non-treated cells (Figures 4.7B and 4.8B). This is the first time as far as we are aware that phosphorylation of p38 MAPK has been shown in response to IV iron preparations clinically utilised in CKD. The data suggests more robust phosphorylation of p38 by IV iron sucrose compared to IV FCM, and thus was consistent, but the fact the sample were blotted on separate gels means that factors related to antibody incubation, washing time, blot exposure or development timing could contribute to differences. However, all blots data was produced using identical protocols. Kinase assay or downstream p38 MAPK substrate phosphorylation can be quantified to confirm these data.

Gathering evidence proposes ROS may be targeted to modulate mitochondrial processes (Ashraf *et al*., 2014). ROS has been linked to the activation of MAPKs such as p38 MAPK pathway, which the latter has been shown to play an important role in the induction of apoptosis in some cell type (Zhang *et al*., 2011; Ashraf *et al*., 2014). Direct exposure of cells to ROS

including, exogenous H_2O_2 to mimic oxidative stress, have been shown in previously published work (Usatyuk *et al*., 2003; Sato *et al.,* 2014) and in our lab to cause activation of p38 MAP kinase, see appendix 1. To confirm that damage to p38 MAPK phosphorylation contributes to damage to mitochondria integrity in IV iron sucrose-treated HUVEC by causing imbalance between the apoptotic proteins, Bcl-2 and Bax, inhibition of p38 MAPK by SB203580 was investigated. Following SB203580 treatment, IV iron sucrose did not cause apoptosis as measured by Annexin V. In addition, less activation in Bcl-2 and Bax occurred, consistent with cell survival by pre-treatment of HUVEC with SB203580.

P38 MAPK is a serine-threonine kinase and was indicated as a potential Bcl-2 kinase (Torcia *et al*., 2001). In addition, Bcl-2 phosphorylation is synchronous with p38 MAPK activation through direct interaction with Bcl-2 protein in the mitochondrial compartment. Furthermore, phosphorylation of Bcl-2 can occur on serine and threonine residues (including Ser^{87} and Thr^{56}) which are located in a non- structured loop between BH3 and BH4 domains (Nencioni *et al*., 2009), results in a decrease in the anti-apoptotic potential of Bcl-2 protein (De Chiara *et al*., 2006). All these can explain how p38 MAPK activation is linked to alteration of Bcl-2 expression and activity. Thus, p38 MAPK activation seems to be essential for the activation of the intrinsic pathway apoptotic signal. Although the mechanism has not been previously investigated, our data shows activation of p38 MAPK is affecting the balance of expression of Bcl-2 and Bax, which are involved in IV iron induced apoptosis, and may potentially lead to release of cytochrome *c* and downstream activation of caspase-3, resulting in apoptosis (Nencioni *et al*.,

2009). These results can be in line with a study by Grethe *et al* (2004) showed that inhibition of p38 MAPK by SB203580 attenuated the apoptosis in EA.hy926 cell line exposed to 10 μM TNF-α. Another study by Yu *et al* (2006) revealed that, 10 μM SB203580 significantly reduced the apoptosis of EC treated with H_2O_2. Additionally, SB203580 also upregulated Bcl-2 and down regulated Bax expression in HUVEC exposed to Lipopolysaccharide (Lim *et al*., 2009). Interestingly, IV FCM phosphorylated p38 MAP kinase but did not induce apoptosis of HUVEC either through direct measurement (TUNEL assay and Annexin V-FITC/PI staining) or indirectly (alteration of Bcl-2 or Bax protein expression). In contrast, IV iron sucrose showed significant induction of apoptosis, activation of p38 MAP kinase and down-regulation of Bcl-2 and up-regulation of Bax protein expression. However, to be completely convinced about the role of p38 MAPK in HUVEC apoptosis in response to IV iron sucrose, molecular methods would be required to confirm these results (see future studies, section 6.1.2).

Our data in chapter 3 clearly showed that IV iron sucrose or IV FCM significantly induced generation of ROS in HUVEC. It has been shown previously that p38 MAPK is upstream of ROS production through elevation of mitochondrial ROS level under hypoxia (Ashraf *et al*., 2014). To investigate whether ROS is playing a key role in p38 MAPK activation in IV iron preparation-treated HUVEC, antioxidant treatment (NAC) was carried out. The use of NAC significantly reduced p38 MAPK phosphorylation, which implicates that ROS must be at least partly responsible for activating this signalling pathway which ultimately plays a role in inducing IV iron sucrose mediated apoptosis. Our data are in line with the observation by Usatyuk *et*

al (2003) who found that, treating bovine lung microvascular ECs (BLMVECs) with 5 mM NAC and 100 μM H_2O_2 significantly reduced the activation of p38 MAPK, which was mediated by oxidative stress induction. In addition, another study by Wang and others (2010) found that, p38 MAPK phosphorylation in response to particulate matter (PM), was attenuated by treating human pulmonary artery EC with 5 mM NAC compared with non-treated cells. This can be explained as IV FCM did not activate p38 MAPK to the same extent as IV iron sucrose. Further discussions on the upstream mechanisms of apoptosis are in chapter 6. All data in this chapter support the view that IV iron compounds may impair EC function resulting in disintegration of vascular structure, increased vascular permeability, proliferation of SMCs and macrophages, and platelet activation and aggregation and, thus can be a key element in the progression and increase death of CKD patients due to CVD.

4.5. CONCLUSION

In conclusion, our study is the first to demonstrate that both IV iron preparations (sucrose and FCM) induced p38 MAPK activation and this activation was partly due to ROS elevation. IV FCM HUVEC did not induce apoptosis, downstream of p38 MAPK, and had no effect on anti-apoptotic/pro-apoptotic protein family members Bcl-2 and Bax. In contrast, IV iron sucrose showed up-regulation of pro-apoptotic Bax and down-regulation of anti-apoptotic Bcl-2 proteins to induce mitochondrial membrane permeabilization and EC apoptosis as measured by TUNEL assay and Annexin V-FITC/PI staining.

CHAPTER 5

THE EFFECT OF IV IRON PREPARATIONS ON HUMAN ENDOTHELIAL CELL ACTIVATION AS DEFINED BY INCREASED ADHESION MOLECULE EXPRESSION

5.1. INTRODUCTION

As already discussed in chapter 1 section 1.1.5, death among patients with CKD due to CVD and accelerated atherosclerosis is 10 to 30 times greater than in general populations (Levey and Eknoyan, 1999; Collado *et al*., 2010). Atherosclerosis is a complex and progressive inflammatory disease, affecting arterial blood vessels and frequently associated with renal disease (O'Hare *et al*., 2004) with plaques are found in approximately 30% of CKD patients (Amann *et al*., 2004). As part of its important role in leucocyte recruitment during inflammation, the endothelium expresses CAMs, including VCAM-1 and ICAM-1, which promote the adhesion and migration of blood cells such as T-lymphocytes and monocytes to attach and migrate into the sub-endothelial space in the intima (Libby *et al.,* 2002). Under physiological conditions, ICAM-1 is constitutively expressed on healthy resting ECs surface, while VCAM-1 is expressed only on the surfaces of activated ECs and certain leukocytes (such as macrophages). Both adhesion molecules can be up-regulated by many cytokines, including TNF-α and IL-1 (Frank and Lisanti, 2008). EC damage or activation as some investigators refers to, as the key mechanism underlying chronic inflammatory conditions, including atherosclerosis, a vascular disease (Sorensen *et al.,* 1994; Gokce *et al*., 2002; Libby *et al.,* 2002). The dysfunction of ECs is a well characterized phenotype that plays an important role not only in the initiation and the development of atherosclerosis, but is also involved in the late stages of CVD by the development of thrombosis that leads to vessel occlusion and acute cardiovascular events (Hansson, 2005). EC dysfunction is characterized by increased vascular permeability and up-regulation of

adhesion molecule expression including ICAM-1, VCAM-1 (Libby *et al*., 2002). *In vitro* experiment with serum from CKD patients resulted in damage of HAEC in culture compared with control, through the inhibition of eNOS which is mediated by serum advanced glycation end product (AGE) from these patients. This is indirect evidence that EC are damaged in these patients (Schmidt *et al*., 1999). In addition, EC dysfunction has been observed in HD patients as well as in children with advanced renal insufficiency (Kari *et al*., 1997; Passauer *et al*., 2000), which provides an essential relation between CKD and the high risk for CV events that patients with CKD exhibit (Félétou and Vanhoutte, 2006; Perticone *et al*., 2010).

The expression of both ICAM-1 and VCAM-1, on ECs is thought to be important in immune and inflammatory diseases, such as atherosclerosis (Price and Loscalzo, 1999). Emerging evidence showed that these molecules are present in the progression of atherosclerotic lesions, which suggests that they play a key role in the pathogenesis of atherosclerotic CVD (Papayianni *et al*., 2002; Rezzani *et al*., 2013), which is considered as the main cause of death in CKD patients. Levels of soluble adhesion molecules, especially sICAM-1 and sVCAM-1 have been consistently shown to be increased in CKD patients (Bonomini *et al*., 1998; Stancanelli *et al*., 2010). A number of *in vivo* studies found that CKD is associated with oxidative stress elevation (Pecoits-Filho *et al*., 2003; Oberg *et al*., 2004). In addition, several studies have reported that elevated levels of serum soluble adhesion molecules were associated with carotid intima media thickness, which is considered as a surrogate marker of early-phase coronary atherosclerosis in patients with CKD (Stenvinkel *et al*., 2002; Papayianni *et al*., 2002). Previous

study found that increased carotid intima media thickness was significantly associated with annual IV iron doses in patients with ESRD, who administered with different doses 50-100 mg of IV iron sucrose weekly for a period of one year (Drüeke *et al*., 2002). The authors also showed that increased carotid intima media thickness was significantly associated with increased oxidative stress marker, AOPP-induced by IV iron sucrose, suggesting that this IV iron preparation play a role in oxidative stress, and consequently can cause an increase in the incidence of atherosclerosis (Drüeke *et al*., 2002; Reis *et al*., 2005). There are different markers can be used *in vivo* to assess EC dysfunction or injury such as, increased plasma levels of EC-generated molecules like von Willebrand factor (vWF), elevated numbers of circulating EC and endothelial progenitor cells as well as vascular activity to an appropriate stimulus (Brevetti *et al*., 2008). An alternative method to assess endothelial activation and dysfunction *in vitro* involves the measurement of adhesion molecules ICAM-1 and VCAM-1 (Papayianni *et al*., 2002). One of the main and most common causes of anaemia is iron deficiency, which is common in CKD patients (Dillon et *al*., 2012). It has been found that, patients who suffer from iron deficiency have higher levels of soluble adhesion molecule expression, which by itself can be a risk factor of atherosclerosis progression (Yuksel *et al*., 2010). Yuksel *and* others suggested that the increased adhesion molecule expression in these patients can be due to increased risk of atherosclerosis as a result of elevated lipid peroxidation. Moreover, IV iron administration can provoke ROS production *in vivo* (Drüeke *et al*., 2002), results we have been able to replicate in our model in chapter 3. ROS under normal conditions, serve as

important physiological intracellular messengers (Hensley *et al*., 2000). However, overproduction of ROS such as H_2O_2 and superoxide anion have been found to induce endothelial activation and dysfunction, through the activation of redox-sensitive transcription factors, including NF-κB which is a key regulator of cytokines, chemokines, and cellular adhesion molecules (DiDonato *et al*., 1997; Lum and Roebuck 2001; Chen *et al*., 2004). Furthermore, IV iron sucrose, showed adverse CV outcomes, leading to increased mortality in CKD patients and this was associated with increased expression of adhesion molecules ICAM-1 and VCAM-1 *in vitro* through activation of NF-κB (Kuo *et al*., 2014). In addition, *in vitro* studies showed that, a number of IV iron preparations including, IV iron sucrose, iron dextran, ferric gluconate, IV FCM and ferric chloride hexahydrate induced the expression of ICAM-1 and VCAM-1 in different cell types, HAEC and PBMC (Kuo *et al*., 2012; Martin-Malo *et al*., 2012; Kuo *et al*., 2014). However, none of these studies looked at the effect of IV FCM on adhesion molecule expression in ECs. Hence, in this chapter, we hypothesized that IV iron preparations might promote EC damage through endothelial adhesion molecule expression and consequently accelerate atherosclerotic lesion progression in patients with CKD.

Therefore, the aim was to investigate the effect of clinically relevant concentrations of IV iron sucrose or IV FCM used in previous chapters on the activation of primary HUVEC by the assessment of EC, ICAM-1 and VCAM-1 gene and protein expression using real time PCR (RT-PCR), quantitative PCR (qRT-PCR) and Western blot methods. Previous work has shown beneficial effects of antioxidants in CKD (Agarwal *et al*., 2004; Swarnalatha

et al., 2010). Therefore, in addition we aimed to investigate whether treatment with NAC reduces the expression of adhesion molecules in IV iron preparations treated HUVEC.

5.2. MATERIALS AND METHODS

5.2.1. Materials

Agarose (15510-027) was purchased from Invitrogen (Paisley, UK). Nuclease-free water (129115) was purchased from Qaigen (Crawley, West Sussex, UK). For total RNA isolation: GenElute™ Mammalian Total RNA Miniprep Kit (RTN10-1KT), DNAse I Amplification Grade (AMPD1-1KT), ethidium bromide (E7637) and recombinant human tumour necrosis factor-alpha (TNF-α, T-0157) were purchased from Sigma-Aldrich (Pool Dorest, UK). For complementary deoxyribonucleic acid (cDNA) synthesis: ImProm-II™ Reverse Transcription System (A3800) and Tris-acetate-EDTA buffer (TAE) buffer (V4271) were purchased from Promega (Soughtampton, UK). DreamTaq Green PCR Master Mix (2X) (K1081) was purchased from Thermo Scientific (Loughborough, UK). For mRNA isolation: GenElute mRNA Miniprep kit (MRN10-1KT) was purchased from Sigma-Aldrich (Pool Dorest, UK).

For RT-PCR reaction, PCR reagents were purchased from Promega (Southampton Science, UK) and used according to the manufacturer's protocol. PCR primers were purchased from Sigma-Aldrich (Pool Dorest, UK). For qRT-PCR, Fast SYBR® Green Master Mix (4385612), MicroAmp® Optical 8-Cap Strips (4323032) and MicroAmp® Fast Optical 96-Well Reaction Plate (4346906) were all purchased from Life Technologies

(Paisley, UK). Real-Time PCR primers include Hs-ICAM1-1 (QT00074900), Hs-VCAM1-1 (QT00018347) and Hs-ACTB (QT00095431), all were purchased from Qiagen (Manchester, UK). Amplification of individual genes was performed on StepOnePlus™ Real-Time PCR Systems.

For Western blot, rabbit polyclonal CD54/ICAM-1 antibody (4915) and rabbit polyclonal VCAM-1 antibody (12367) were purchased from New England Biolabs, UK. Anti-GAPDH antibody (ab9485) was obtained from Abcam, Cambridge, UK.

5.2.2. Methods

5.2.2.1. Assessment of ICAM-1 and VCAM-1 mRNA gene expression in IV iron preparation-treated HUVEC

5.2.2.2. Total RNA extraction from cultured HUVEC

Total RNA was isolated from differently treated and non-treated HUVECs using GenElute mammalian total RNA Miniprep kit (Sigma-Aldrich, UK). Cells were grown in 6 well plates at a density of $2x10^5$ cells per well. Upon confluence, HUVEC were treated as described before with 50 µg/ml of IV iron sucrose or IV FCM or left untreated, which were considered as control. Subsequently, cells were washed twice with RNase-free PBS and then cells were detached from plates by Trypsin/EDTA (0.05%0.02%). Afterwards, cells were collected by scraping into 1 ml RNA lysis buffer. Cells were homogenized by passing the lysate at least 5 times through a blunt 20-gauge needle (0.9 mm diameter) (NV-2025R, Terumo, Leuven, Belgium) fitted to an RNase-free syringe (BD Plastipak). The homogenate was then transferred to a GenElute filtration column and centrifuged (Eppendorf 5415 R) at 13000x g

for 2 min to remove all cellular debris and to shear the DNA. After centrifugation, the filtration column was discarded, and an equal volume of 70% ethanol (made using 0.05% (v/v) DEPC treated water) was added to the filtered lysate and mixed thoroughly by vortexing. Afterwards, the combination of lysate/ethanol mixture was then transferred to a clear GenElute binding column and centrifuged for 15 seconds at 13000x g to bind RNA to the column. The flow through was then discarded, and the binding column with the bound total RNA moved back to the collection tube. The remaining sample was added to the binding column, and the same procedure repeated. The binding column was then transferred to a new collection tube. The binding column was washed to remove contaminants; 500 µl of wash solution 1 was added to the binding column and centrifuged for 15 seconds at 13000x g. The column was transferred to a new collection tube and a second wash carried out by adding 750 µl wash solution 2 to the binding column and centrifuging for 2 min at 13000x g, the flow-through was discarded. The column was transferred to a new collection tube to elute the RNA from the binding column; 50 µl elution solution was added to the centre of the binding column and centrifuged for 1 min at 13000x g. The flow through containing the purified total RNA was collected, and checked immediately for quality, purity and concentration before RNA extraction for each sample, or stored at -80°C until required.

5.2.2.3. Isolation of mRNA from primary HUVEC

mRNA was isolated from the total RNA samples using a GenElute mRNA Miniprep kit (Sigma-Aldrich, UK) in accordance with the manufacturer's instructions provided. The volume of each total RNA sample was brought up

to 250 µl using RNase-free water, then mixed with 250 µl 2X Binding solution and 15 µl oligo (dT) beads, and vortexed thoroughly. The samples were incubated at 70^0C for 3 min to denature RNA and were then left at room temperature for 10 min. Samples were then centrifuged for 2 min at 13000x g to pellet the beads. The supernatants were discarded and the pellets re-suspended in 500 µl wash solution 1 by vortexing. Each sample suspension was then transferred to a spin filter/collection tube and centrifuged for 2 min at 13000x g, before the flow through was discarded and the column containing the beads: mRNA complex was washed again with a second 500 µl wash solution 2 followed by centrifugation for 2 min at 13000x g. The column was transferred into a fresh collection tube and 50 µl elution solution (preheated to 70^0C) was added to the centre of the filter and incubated for 5 min at 70^0C, before centrifuging for 1 min at 13000x g. This elution procedure was repeated with an additional 50 µl elution solution. The isolated mRNA was then stored at -80^0C until further use or kept on ice for immediate cDNA synthesis.

5.2.2.4. Measurement of quality and quantity of isolated total RNA by spectrophotometer

The quality and quantity of RNA in each sample was spectrophotometrically checked. The spectrophotometer was first calibrated to zero using nuclease-free water in a quartz cuvette. Then, RNA was diluted in RNase-free water (1:20) and then the UV absorbance was detected using a Jenway 6305 UV/VIS spectrophotometer (Keison product, Essex, UK) at two different wavelengths: 280 nm for DNA and 260 nm for RNA.. RNA purity was evaluated by calculating the ratio (absorbance at 260 nm/absorbance at 280

nm) and RNA quality was considered good if the ratio was between (1.8-2.2). RNA concentration was determined by using the equation where Ab 260 is the absorbance at 260 nm and DF is the dilution factor:

RNA concentration (µg/ml) = Ab 260 x DF x 40

5.2.2.5. Agarose gel electrophoresis

A 1.5-2.0% (w/v) gel was formed by melting agarose in 1 x TAE using a 950W microwave (Proline Micro chef ST44) on power 7 for 180 seconds till the agarose was completely dissolved. Subsequently, the solution was left at room temperature to cool down to approximately 50-60^{0}C and then ethidium bromide was added to the solution and mixed (final concentration of 0.6 µM). The solution was poured into plastic gel trays containing gel combs to form wells, and left for approximately 45 min to set. Subsequently, gel combs were removed gently and gel was placed in an electrophoresis tank containing enough 1 x TAE buffer to cover the gel. 8 µl of RNA for each sample was mixed with 2.5 µl loading dye and carefully loaded into the gel. The tank set to run at 100V for approximately 30 min. Subsequently, the UVitec gel documentation system (UVItec Limited, Cambridge, UK) was used to visualise total RNA and the images were captured using software UVitec version 12.06 for windows.

5.2.2.6. Deoxyribonuclease I (DNase I) treatment

Amplification grade DNase I (Sigma-Aldrich, UK), was used to eliminate any contaminating genomic DNA from the isolated RNA prior to cDNA synthesis by reverse transcription. A 0.5 ml Eppendorf tube (Alpha Laboratories, UK) was used to make each reaction mix. This consisted of 8 µl of RNA, 1 µl

manufacturer supplied reaction buffer and 1 µl DNase I amplification grade which were incubated for 15 min at room temperature. Subsequently, DNase I was inactivated by adding 1 µl EDTA (25mM; Invitrogen Ltd), and then the reaction was heated to 65^0C for 10 min. The resulting DNA-free RNA samples were either placed on ice to be used immediately for cDNA synthesis or stored at -80^0C until further use.

5.2.2.7. Complementary DNA synthesis

The purified RNA was reverse transcribed to cDNA, which is ideal for PCR experiments. ImProm-II™ Reverse Transcription System was used. The protocol recommended by the manufacturer was followed. All reagents and RNA samples were thawed on ice and then any unused portion returned to -80^0C as soon as aliquots were taken. Briefly, 2 µl RNA (1 µg/ml) was mixed with 1 µl Oligo (dT) primer (0.05 µg/µl), denatured by incubating at 70^0C for 10 min and thereafter placed on ice for 5 min. The reaction mix for first strand synthesis, including 2 µl of 10X ImProm-II™ reaction buffer, 4 µl $MgCl_2$ (25 mM), 2 µl dNTPs Mix (10 mM), 0.5 µl recombinant RNase in Ribonuclease Inhibitor (40 units/µl), 0.5 µl of avian myeloblastosis virus (AMV) reverse transcripts (high concentration: 25 units/µl). The first strand synthesis reaction was incubated at 42^0C for 60 min to allow cDNA synthesis from mRNA by reverse transcription and then at 90^0C for 5 min to inactivate the reverse transcriptase, then volume was made up to 80 µl by Nuclease-free water. Produced cDNA was aliquoted and maintained at -20^0C for long term storage. Then, β-actin gene was amplified by RT-PCR to ensure synthesis of the first-strand of cDNA, had been successfully and validation of PCR condition.

5.2.2.8. Primer design

A web-based genome database (www.ncbi.nlm.nih.gov) was used to analyse the genomic sequences and protein of the target genes of interest (β-actin, ICAM-1 and VCAM-1) to facilitate the design primers for PCR. For each gene, pair of primers (forward and reverse) were checked for the purity and specificity to the target gene intended via common used software, primer3plus (www.bioinformatics.nl). Primers were then ordered from Sigma-Aldrich (Pool Dorset, UK), and were re-suspended to a final concentration of (100 ng/µl) in nuclease free water.

Table 5.1: The sequences and product size of ICAM-1, VCAM-1 and β-actin primers. The primer sequences, their optimised RT-PCR conditions and their expected amplicon size are showed below, where F is the forward primer and R is the reverse primer.

Gene of interest	Sequence of primers	Expected product size (bps)
β-actin	**F: 5'AAATCTGGCACCACACCTTC3'** **R: 5'CTCCTTAATGTCACGCACGA3'**	**392**
ICAM-1	**F: 5'GCAAGAACCTTACCCTACGC3'** **R: 5'TGATCTCTCCTCACCAGCAC3'**	**154**
VCAM-1	**F: 5'TTTCTGGAGGATGCAGACAG3'** **R: 5'TACAGCCTGCCTTACTGTGG3'**	**106**

5.2.2.9. RT-PCR for detection of ICAM-1 and VCAM-1 mRNA expression in HUVEC

All reagents were thawed on ice and then, RT-PCR was performed using 1 µl to 5 µl cDNA, 17 µl of nuclease free water, 2.5 µl of 10 X ImProm-II™ reaction buffers, 1.5 µl $MgCl_2$ (25 mM). In order to get the best amplification during PCR, each target primer set required optimization of primer concentrations and annealing temperatures. The PCR thermocycling program was: initial denaturation at 94^0C for 30 seconds, followed by 30 cycles of: PCR amplification at 94^0C, optimum annealing temperature at 58-63^0C for 20 seconds, extension at 72^0C for 1 min, followed by a final 10 min extension step at 72^0C. After the thermocycling finished the tubes were cooled at 4^0C. The PCR products were placed on ice to be analysed immediately by agarose gel electrophoresis or could be stored at -20^0C for later analysis.

5.2.2.10. Quantitative real time PCR (qRT-PCR) for detection of ICAM-1 and VCAM-1 mRNA expression in HUVEC

QRT-PCR was carried out to quantify mRNA expression of ICAM-1 and VCAM-1 in control or IV iron-treated HUVEC. PCR reaction was placed in MicroAmp® Fast Optical 96-Well Reaction Plate (Applied Biosystem, UK) and performed using StepOnePlus™ Real-Time PCR Systems (Applied Biosystem, UK). All reagents were thawed on ice and then, any unused portion returned to the freezer at -20^0C as soon as aliquots were taken. PCR reaction mix was prepared in 0.5 ml Eppendorf tubes (Alpha Laboratories) on ice by mixing all of the following reagents: 5 µl Fast SYBR® Green Master Mix, 2 µl (10 µM) oligo primers, 2 µl cDNA and then the volume was made up

to 15 µl by adding 6 µl RNase-free water. Samples were prepared in duplicate and the plate was centrifuged briefly to ensure a good mix of reagents. The thermocycler parameters were 50^0C for 2 min and 95^0C for 20 seconds, followed by 50 cycles of 95^0C for 15 seconds and 60^0C for 30 seconds. The data obtained from each reaction was analysed by StepOne™ Software v2.2.2. Relative quantification representing the change in gene expression from real-time quantitative polymerase chain reaction between experimental groups was calculated by comparative C_T method. The data was analysed by calculating the relative quantification (RQ) using the equation: RQ = $2^{-\Delta CT}$ X100, where ΔC_T = C_T of target gene− C_T of endogenous gene (housekeeping gene). Evaluation of $2^{-\Delta CT}$ indicate the fold change in gene expression, normalized to the internal control (β-actin) which enable the comparison between differently treated cells. All results then were logged to the base 10 ($Log_{10}x$) and a ratio paired *t*-test was applied to detect the significant level of the difference between differently treated cells.

5.2.2.11. Detection of ICAM-1 and VCAM-1 protein expression in HUVEC by Western blot

The protocol was followed as described in previous section (4.2.1.6). Briefly, for ICAM-1 protein analysis, following SDS PAGE separation and Western blotting , the PVDF membrane was blocked with blocking buffer as described in section (4.2.1.6), and incubated with rabbit polyclonal CD54/ICAM-1 antibody (1:2000, New England Biolabs, UK). For VCAM-1 protein analysis, the membrane was incubated with rabbit polyclonal VCAM-1 antibody (1:2000, New England Biolabs, UK). Membranes were then incubated with horseradish peroxidise HRP- conjugated donkey anti rabbit IgG (1:5000).

GAPDH (1:5000, Abcam, UK) was used as internal control. The diluent used to dilute all antibodies was TBS-T including 2% (w/v) BSA. All Western blotting experiments were performed on at least 3 independent samples from different donors and densitometric analysis of ECL exposure was performed using Image J software. The results were expressed in relative densitometry compared with GAPDH.

5.3. RESULTS

5.3.1. The effect of IV iron preparations on ICAM-1 and VCAM-1 mRNA expression in HUVEC

5.3.1.1. Efficiency of isolated RNA

Total RNA was isolated from HUVECs as described in section 5.2.2.2. and the efficiency of the purification method was assessed by running the isolated RNA on 1.5% agarose gel. The image in Figure 5.1 shows RNA isolated from seven different samples including non-treated cells, IV iron sucrose or IV FCM-treated cells at 2, 6 and 24 hr. Two main bands for each sample could be identified representing the two ribosomal RNA (rRNA) components 18S and 28S. 28S rRNA band was approximately twice as intense as the 18S rRNA band. This indicates that isolated RNA was of good quality as intact RNA has these two bands with a 2:1 ratio (28S:18S). Degraded RNA will not exhibit 2:1 ratio and will appear as a very low molecular weight smear (Skrypina *et al*., 2003).

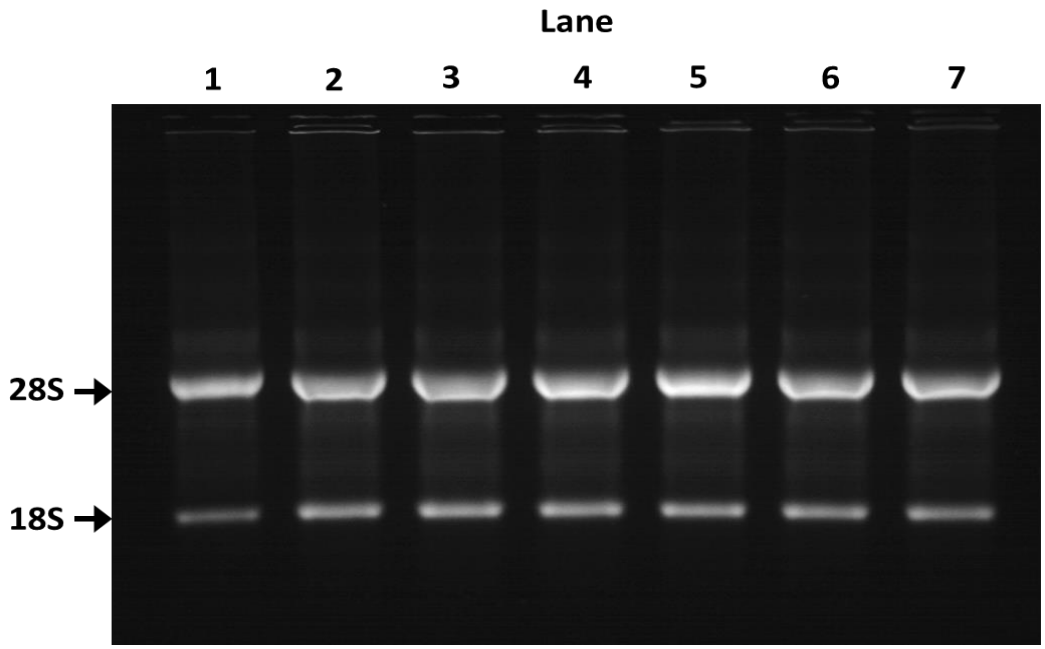


Figure 5.1: Representative image showing the quality of isolated RNA. Total RNA was separated on 1.5-2.0% (w/v) agarose gel in 1 x TAE and allowed to run at 100V for approximately 45 min. Two bands represented two rRNA compounds 18S and 28S, were obvious in the total RNA samples, exhibiting a 2:1 ratio which confirmed good quality of RNA. **Lane 1:** Non-treated cells (control), **lanes 2, 4 and 6:** HUVEC treated with 50 µg/ml IV iron sucrose at 2, 6 and 24hr respectively, **lanes 3, 5 and 7:** HUVEC treated with 50 µg/ml IV FCM at 2, 6 and 24hr respectively. The image represents results of 3 different donors (n=3).

5.3.1.2. RT-PCR for ICAM-1 and VCAM-1 mRNA gene expression in HUVEC

ICAM-1 and VCAM-1 mRNA expression was assessed using RT-PCR in non-treated and differently treated HUVEC from three different patients, and TNF-α treated cells (where ICAM and VCAM gene expression up-regulation is well established in our lab and others) were used as positive control. β-actin primer was used as housekeeping gene for variations in PCR product levels due to any differences in RNA isolation efficiency and PCR reaction efficiency. In addition, variations in the numbers of cells between differently treated wells due to the effect of IV iron preparations sucrose or IV FCM on cell proliferation were adjusted by normalizing the results of each sample against its own level of β-actin mRNA as β-actin is expressed constitutively and not affected by IV iron preparations. Having loaded equal amounts of RNA in the PCR reaction, it can be concluded that β-actin mRNA is expressed constitutively and is not affected by IV iron preparations treatment (Figures 5.2B and 5.3B). All cDNA samples from differently treated cells produced PCR products which corresponded to the expected amplicon size (154bp: ICAM-1, 106bp: VCAM-1 and 392bp: β-actin), and Figure 5.2 shows bands of ICAM-1 and β-actin and Figure 5.3 shows bands of VCAM-1 and β-actin mRNA respectively from three different donors. The RT-PCR analysis showed that ICAM-1 mRNA gene expression was detected in non-treated HUVEC, which confirm that ICAM-1 is constitutively expressed on the healthy and resting HUVEC surface (Frank and Lisanti, 2008). On the other hand, ICAM-1 mRNA gene expression was increased in cells treated by 50 µg/ml IV iron sucrose or IV FCM in a time-dependent manner (2, 6 and 24hr)

(Figure 5.2A). In contrast, Figure 5.3A showed that VCAM-1 mRNA gene expression was not detected in non-treated HUVEC. However, in cells treated with 50 µg/ml IV iron sucrose or IV FCM, VCAM-1 mRNA was detected and elevated in a time dependent manner (2, 6 and 24hr) dependent on type of IV iron preparation used. This indicates that VCAM-1 is not expressed on resting healthy HUVEC surface, but expressed only on activated HUVEC. Figures 5.2A and 5.3A showed significant detection of ICAM-1 and VCAM-1 mRNA expression respectively after stimulation with the proinflammatory mediator TNF-α (120U/ml; 6hr) as a positive control. RT-PCR technique only showed the qualitative of ICAM-1 or VCAM-1 mRNA gene expression in HUVEC. Therefore, qRT-PCR technique was applied to measure and to quantify the differences in ICAM-1 or VCAM-1 mRNA gene expression in non-treated and IV iron sucrose or IV FCM-treated cells.

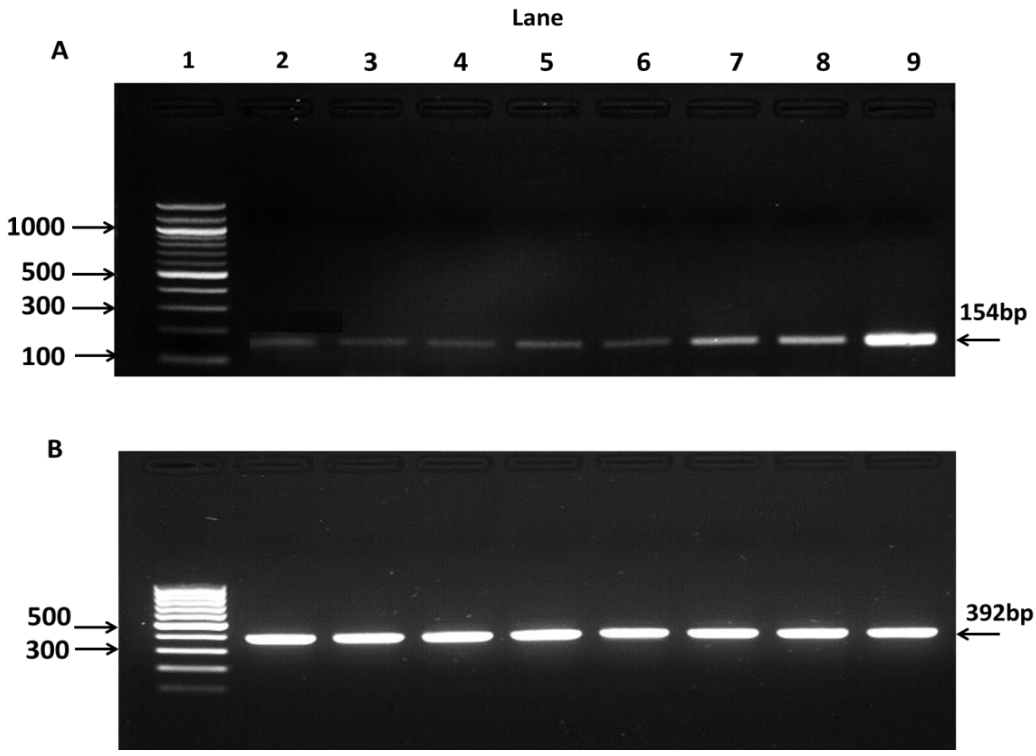


Figure 5.2: The effects of IV iron preparations on ICAM-1 mRNA expression by RT-PCR. 20 µl of the resultant PCR product from each sample were loaded on 1.5% (w/v) agarose gel electrophoresis in 1 x TAE and set to run at 100V for approximately 45 min. TAE- agarose gel photographs showing ethidium bromide stained RT-PCR products of ICAM-1 and β-actin mRNA in HUVEC. **(A):** ICAM-1 mRNA gene expression in HUVEC was investigated by RT-PCR using specific ICAM-1 primer. **(B):** β-actin mRNA gene expression in HUVEC was investigated by RT-PCR using specific β-actin primer. The expected amplicon size is 154bp (A) and 392bp (B) for ICAM-1 and β-actin respectively. **Lane 1:** 10 µl molecular weight markers (DNA ladder 100-1500bp), **lane 2:** Non-treated cells (control), **lanes 3, 5 and 7:** HUVEC treated with IV iron sucrose at 2, 6 and 24hr respectively, **lanes 4, 6 and 8:** HUVEC treated with IV FCM at 2, 6 and 24hr respectively, **lane 9:** TNF-α (120U/ml; 6hr) (positive control).

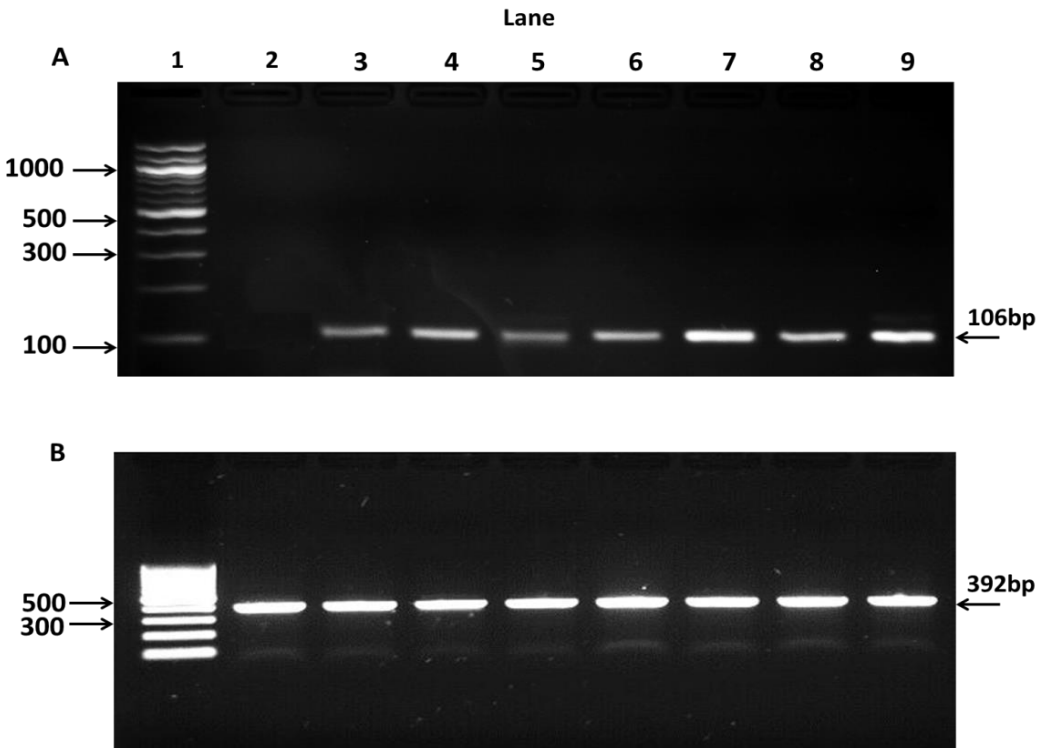


Figure 5.3: The effects of IV iron preparations on VCAM-1 mRNA expression by RT-PCR. 20 µl of the resultant PCR product from each sample were loaded on 1.5% (w/v) agarose gel electrophoresis in 1 x TAE and set to run at 100V for approximately 45 min. TAE- agarose gel photographs showing ethidium bromide stained RT-PCR products of VCAM-1 and β-actin mRNA in HUVEC. **(A):** VCAM-1 mRNA gene expression in HUVEC was investigated by RT-PCR using specific VCAM-1 primer. **(B):** β-actin mRNA gene expression in HUVEC was investigated by RT-PCR using specific β-actin primer. The expected amplicon size is 106bp (A) and 392bp (B) for VCAM-1 andβ-actin respectively.. **Lane 1:** 10µl molecular weight markers (DNA ladder 100-1500bp), **lane 2:** Non-treated cells (control), **lanes 3, 5 and 7:** HUVEC treated with IV iron sucrose at 2, 6 and 24hr respectively, **lanes 4, 6 and 8:** HUVEC treated with IV FCM at 2, 6 and 24hr respectively, **lane 9:** TNF-α (120U/ml; 6hr) (positive control).

5.3.1.3. Detection of ICAM-1 and VCAM-1 mRNA gene expression in HUVEC by qRT-PCR

In order to quantify the levels of mRNA expression of ICAM-1 and VCAM-1 in IV iron sucrose or IV FCM-treated HUVEC, qRT-PCR was carried out. All results were adjusted to the internal housekeeping gene β-actin. Figure 5.4 shows the numbers of PCR amplifying cycles required for each gene to reach a fixed threshold (C_T value). In addition, Figure 5.5 represents graph of melt curve versus temperature in qRT-PCR for ICAM-1, VCAM-1 and β-actin mRNA gene expression, which used as an indicator that only one product is amplified and to show that there are no nonspecific amplification products, such as primer dimers. The results showed that IV iron sucrose or IV FCM induced significant increases in the level of ICAM-1 and VCAM-1 mRNA gene expression in HUVEC. IV iron sucrose or IV FCM induced a slight increase in the level of ICAM-1 mRNA at 2hr. This increase was not significant compared with non-treated cells even when a ratio paired *t*-test was applied (Figure 5.6). In contrast, HUVECs treated with IV iron sucrose or IV FCM for 6 and 24hr increased the expression of ICAM-1 mRNA gene expression compared with non-treated cells. This increase was statistically significant for IV iron sucrose-treated cells at 6 and 24hr ($p<0.01$ and $p<0.001$ respectively), and $p<0.01$ for IV FCM-treated cells at both 6 and 24hr (Figure 5.6). Furthermore, there was a significant increase in ICAM-1 mRNA gene expression in cells treated with IV iron sucrose compared with IV FCM-treated cells at 24hr $p<0.05$ (Figure 5.6). In addition, qRT-PCR showed that, level of VCAM-1 mRNA gene expression for IV iron sucrose or IV FCM-treated cells at 2hr was also slightly increased. However, this

increase was not statistically significant compared with non-treated cells (Figure 5.7). On the other hand, HUVEC treated with IV iron sucrose or IV FCM at 6 and 24hr showed clear increases in VCAM-1 mRNA expression compared with non-treated cells. Ratio paired *t*-test was applied and indicated that the increase in VCAM-1 mRNA was significant for both compounds at 6 and 24hr compared with non-treated cells ($p<0.01$) and ($p<0.001$) respectively (Figure 5.7).

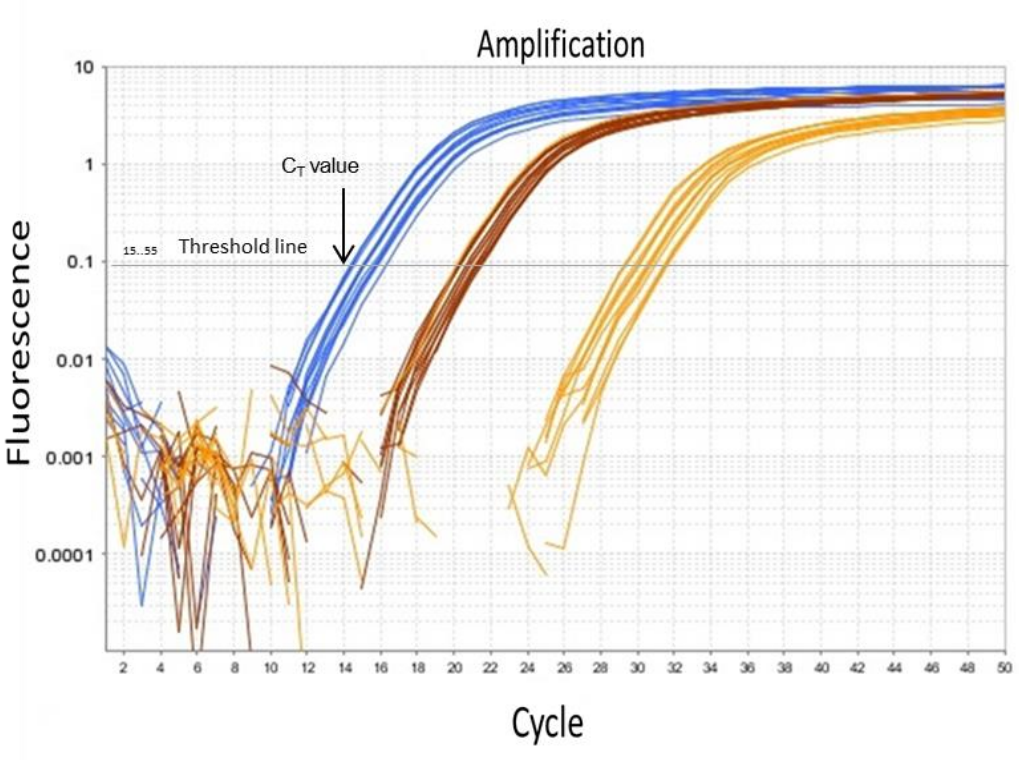


Figure 5.4: Graph shows the amplification curves and the number of qRT-PCR amplification cycles required for each gene to reach fixed threshold fluorescence. The genes which were amplified are: β-actin (blue), ICAM-1 (red) and VCAM-1 (orange).

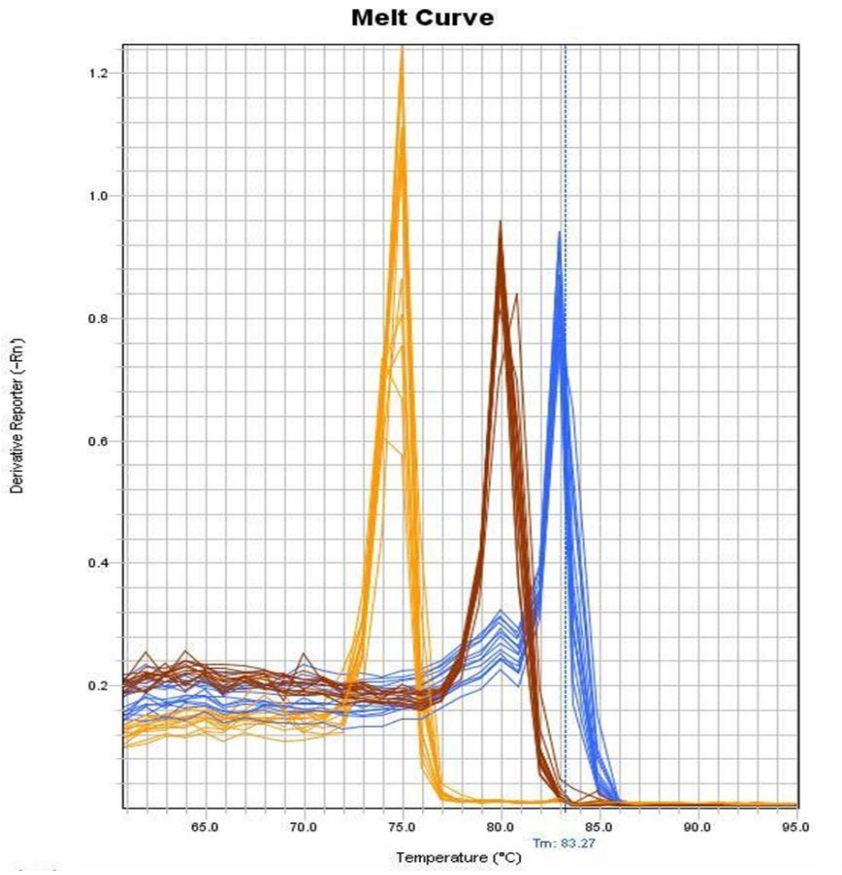


Figure 5.5: Representative graph of melt curve versus temperature in qRT-PCR. The genes which were amplified are: β-actin (blue), ICAM-1 (red) and VCAM-1 (orange).

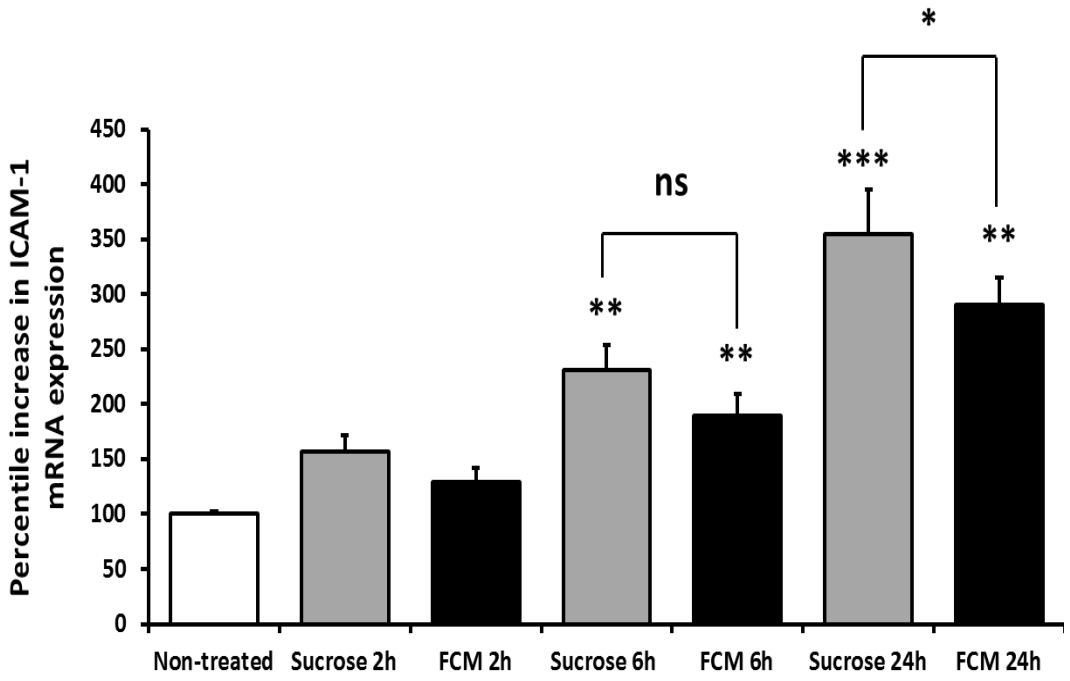


Figure 5.6: The effect of IV iron sucrose or IV FCM on the ICAM-1 mRNA expression in HUVECs by qRT-PCR**.** ICAM-1 mRNA was amplified in differently treated cells using qRT-PCR. HUVECs were grown in complete endothelial growth medium either non-treated or treated with 50 µg/ml IV iron sucrose or IV FCM for the times indicated. Non-treated values were defined as 100% and other values were adjusted accordingly. Data are expressed as mean (+/-SEM) from 3 different donors (n=3). **p<0.01 and ***p<0.001 compared with non-treated cells. *p<0.05 for IV iron sucrose at 24hr versus IV FCM- treated cells at 24hr.

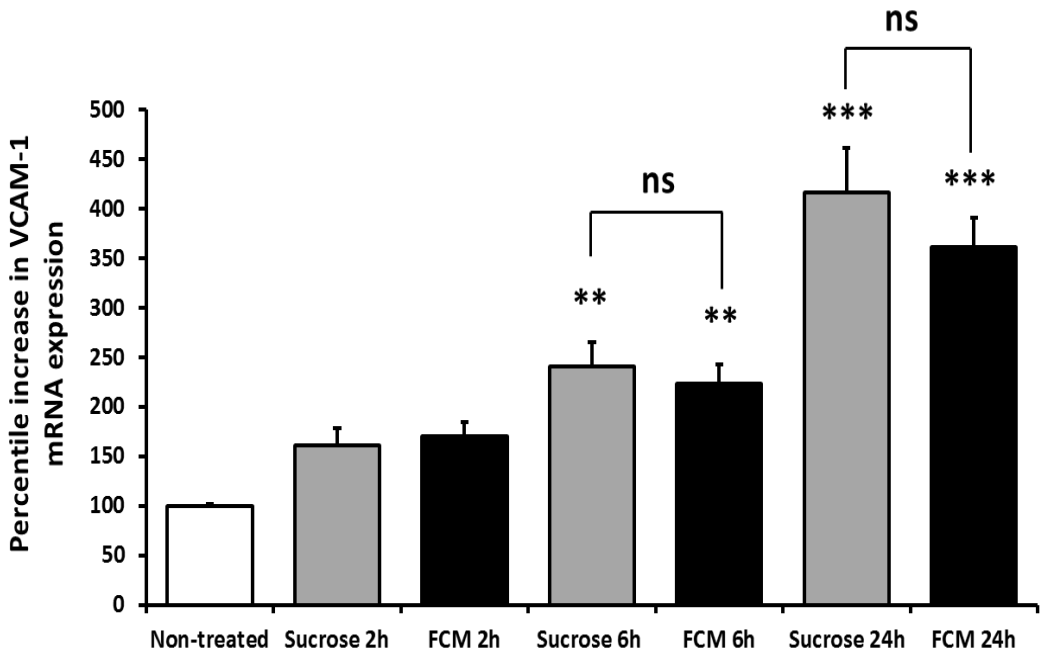


Figure 5.7: The effect of IV iron sucrose or IV FCM on the VCAM-1 mRNA expression in HUVECs by qRT-PCR. VCAM-1 mRNA was amplified in differently treated cells using qRT-PCR. HUVECs were grown in complete endothelial growth medium either non-treated or treated with 50 µg/ml IV iron sucrose or IV FCM for the times indicated. Non-treated values were defined as 100% and other values were adjusted accordingly. Data are expressed as mean (+/-SEM) from 3 different donors (n=3). ** p<0.01 and *** p<0.001 compared with non-treated cells.

5.3.1.4. Detection of ICAM-1 and VCAM-1 protein expression using Western blot

5.3.1.4.1. The effects of IV iron preparations on ICAM-1 and VCAM-1 protein expression

In order to determine whether treatment of HUVEC by IV iron sucrose or IV FCM resulted in increased endothelial ICAM-1 and VCAM-1 protein expression, Western blotting was conducted.

Figure 5.8 A shows a protein band of 90 kDa representing ICAM-1 after HUVEC treatment with IV iron sucrose and IV FCM at concentration of 50 µg/ml across a 24 hour time course (2, 6 and 24hr). HUVEC grown in complete endothelial growth media which was considered as a negative control showed a less intense band compared with the IV iron treated cells confirming that HUVEC express ICAM-1 under basal conditions (Figure 5.8A). HUVECs grown in media containing 50 µg/ml IV iron sucrose or IV FCM at 2hr showed some increase in ICAM-1 protein expression compared to control (Figure 5.8B). However, this increase was not statistically significant. In contrast, IV iron sucrose or IV FCM at 50 µg/ml for 6hr and 24hr showed a significant increase in ICAM-1 expression compared with non-treated HUVEC, IV iron sucrose or IV FCM at 6hr compared with non-treated (p<0.05), IV FCM at 24hr compared with non-treated cells (p<0.01) and IV iron sucrose at 24hr compared with non-treated cells p<0.001 (Figure 5.8B). Furthermore, there was a significant increase in ICAM-1 protein expression in cells treated with IV iron sucrose compared with IV FCM-treated cells at 24hr p<0.05 (Figure 5.8)

Figure 5.9A shows a representative blot with a protein band of 95 kDa corresponding to VCAM-1. VCAM-1 was not detected in non-treated HUVEC as shown in (Figure 5.9A). In contrast, the protein expression of VCAM-1 was detected in HUVEC treated with iron sucrose at 2hr or IV FCM at 2hr and 6hr (Figure 5.9A), however this increase was not significant compared with non-treated cells (Figure 5.9B). On the other hand, cells treated with iron sucrose at 6hr or IV FCM at 24hr showed clearly a significant increase versus non-treated cells ($p<0.05$ and $p<0.01$) respectively (Figure 5.9B). In addition, HUVEC treated with iron sucrose at 24hr showed significant increase in VCAM-1 protein expression compared with non-treated cells ($p<0.001$) (Figure 5.9B). In addition, there was a significant increase in VCAM-1 protein expression in cells treated with IV iron sucrose at 6 and 24hr compared with IV FCM-treated cells at 6 and 24hr $p<0.05$ respectively (Figure 5.9).

Pre-treatment of HUVEC with 2 mM NAC was used to study the effects of a ROS scavenger/anti-oxidant on IV iron sucrose or IV FCM-induced ICAM-1 and VCAM-1 protein expression, to determine if this response was oxidative stress dependent. HUVECs were treated with either IV iron sucrose or IV FCM alone or in the presence of an experimentally relevant concentration of NAC (2mM). Figures 5.10 A and B showed that 2 mM NAC alone had no effect on ICAM1-1 protein expression. However, cells treated with IV iron sucrose or IV FCM and NAC together at 24hr showed significant reduction in the expression of ICAM-1 protein compared with cells treated with IV iron sucrose or IV FCM alone at 24hr ($p<0.01$) (Figure 5.10 A and B). Figure 5.10 A and B showed a significant reduction in ICAM-1 protein expression in cells

co-treated with 2 mM NAC and 50 µg/ml IV iron sucrose at 24hr compared with IV iron sucrose-treated HUVEC alone $p<0.05$. Similar to ICAM-1, 2 mM NAC alone had no effect on VCAM1-1 protein expression on cells treated only with NAC (Figure 5.11 A and B). In contrast, VCAM-1 protein expression in HUVEC cells treated with IV iron sucrose or IV FCM and NAC together at 24hr was significantly decreased compared with IV iron sucrose or IV FCM-HUVEC treated cells at 24hr $p<0.01$ (Figure 5.11 A and B). In addition, VCAM-1 protein expression was significantly reduced in HUVEC co-treated with 2 mM NAC and 50 µg/ml IV iron sucrose at 24hr compared with IV iron sucrose-treated HUVEC alone $p<0.05$ (Figure 5.11 A and B).

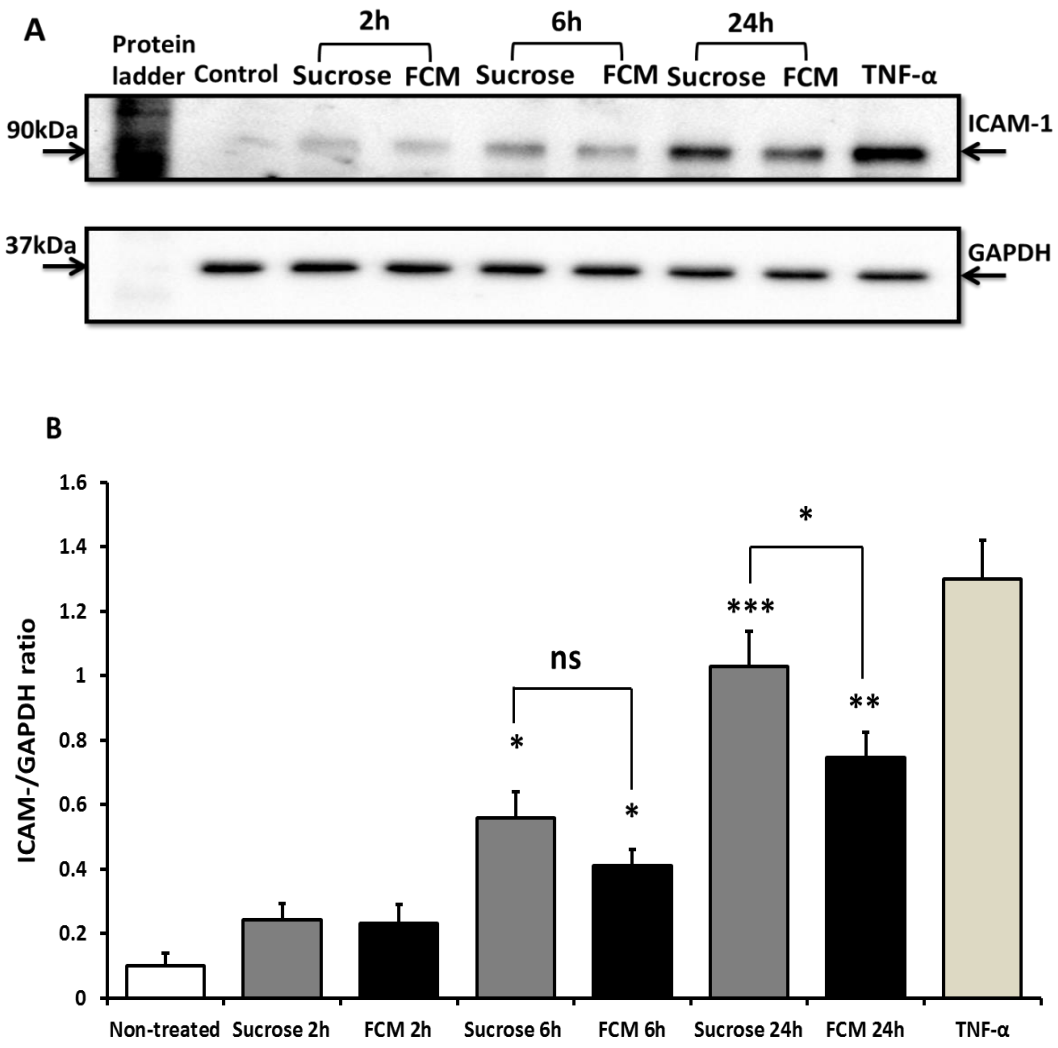


Figure 5.8: The effect of IV iron preparations on ICAM-1 expression in HUVECs by Western blot. HUVECs were incubated with or without 50 µg/ml IV iron sucrose or IV FCM for 2, 6 and 24hr. TNF-α (120U/ml, 6hr) was used as a positive control. **(A):** shows a representative blot indicating a protein band of 90 kDa representing ICAM-1. **(B):** data (mean ± SEM) are shown as a bar graph of densitometry data from 3 different donors (n=3). **p*<0.05, ***p*<0.01 and ****p*<0.01 compared with non-treated HUVECs. **p*<0.05 for IV iron sucrose at 24hr versus IV FCM- treated cells at 24hr.

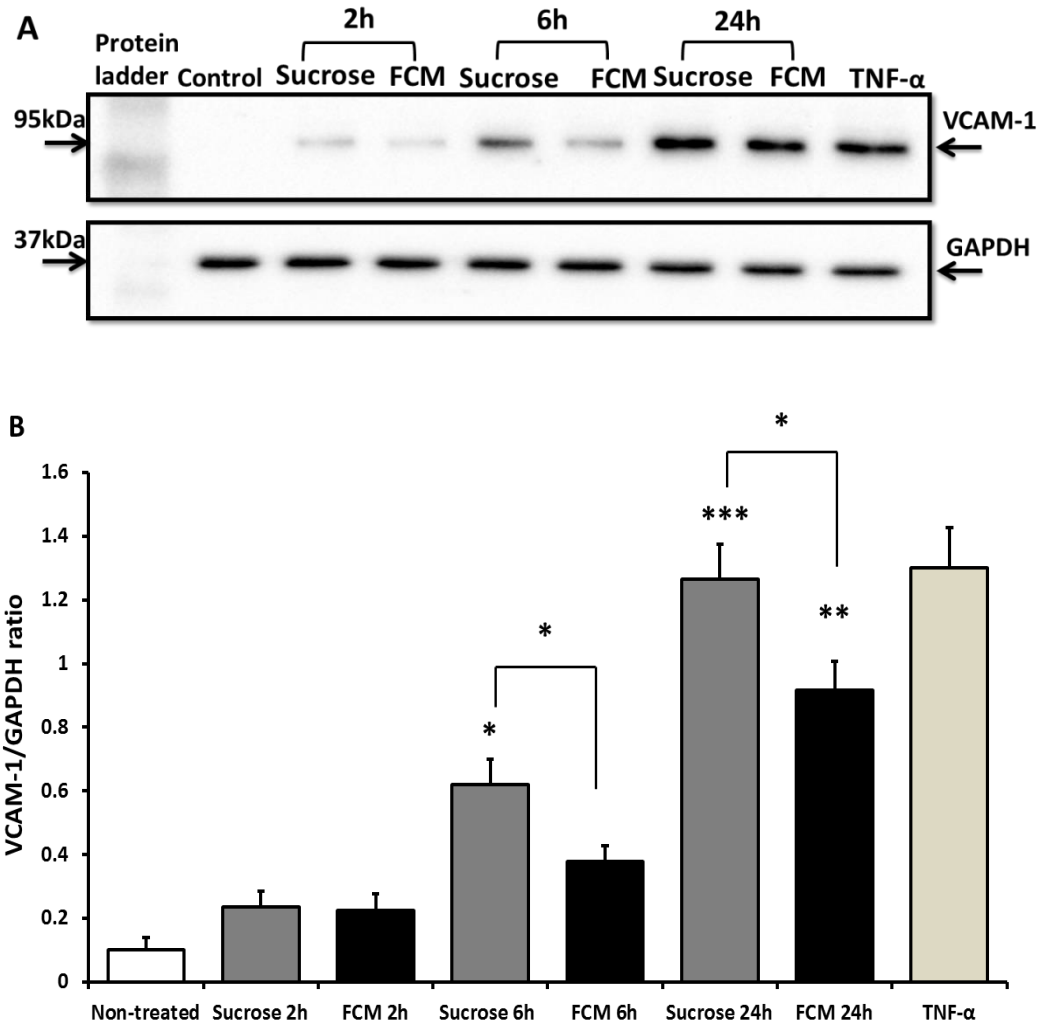


Figure 5.9: The effect of IV iron preparations on VCAM-1 expression in HUVECs by Western blot. HUVECs were incubated with or without 50 µg/ml IV iron sucrose or IV FCM for 2, 6 and 24hr. TNF-α (120U/ml, 6hr) was used as a positive control. **(A):** shows a representative blot indicating a protein band of 95 kDa representing VCAM-1. **(B):** data (mean ± SEM) are shown as a bar graph of densitometry data from 3 different donors (n=3). **p*<0.05, ***p*<0.01 and ****p*<0.01 compared with non-treated HUVECs. **p*<0.05 for IV iron sucrose at 24hr versus IV FCM- treated cells at 24hr.

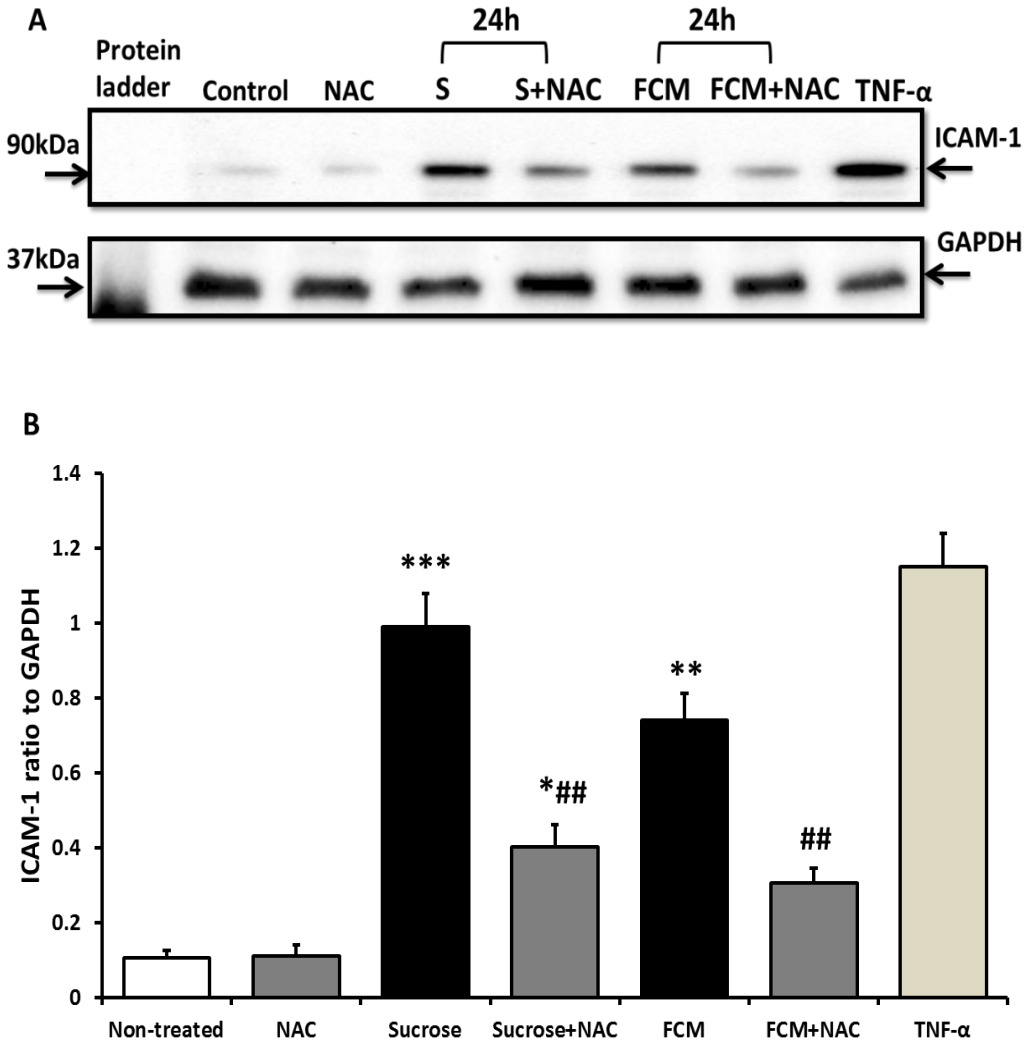


Figure 5.10: The effect of NAC co-incubation on ICAM-1 protein expression in iron-stimulated HUVECs by Western blotting. HUVECs were incubated with or without 50 µg/ml IV iron sucrose or IV FCM and 2 mM NAC for 24hr. TNF-α (120U/ml, 6hr) was used as a positive control. **(A):** show protein band of 90 kDa representing ICAM-1 and (S) represents iron sucrose. **(B):** data (mean ± SEM) are shown as a bar graph of densitometry data from 3 different donors (n=3). *p<0.05, **p<0.01 and ***p<0.01 compared with non-treated cells. $^{\#\#}p$<0.01 for IV iron sucrose or IV FCM and NAC together at 24hr compared with IV iron sucrose or IV FCM alone at 24hr.

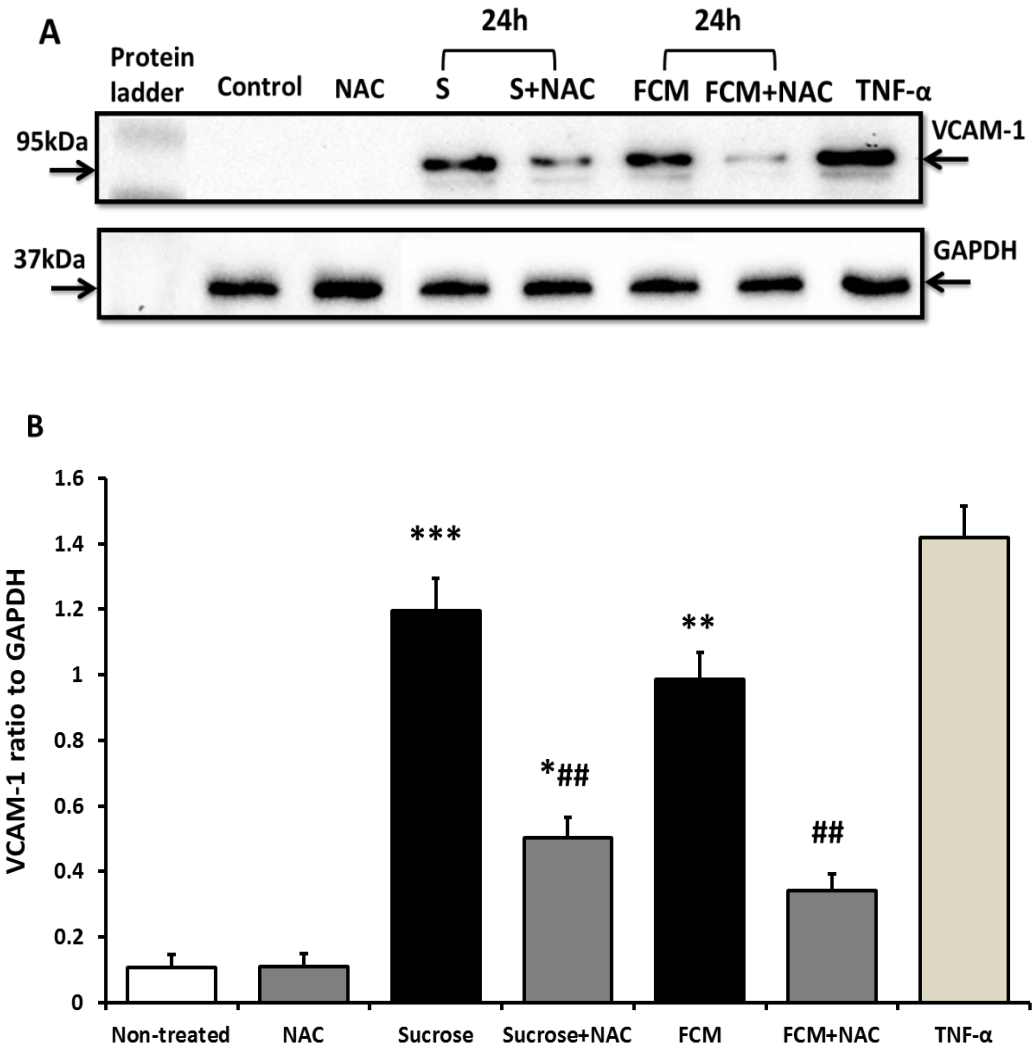


Figure 5.11: The effect of NAC co-incubation on VCAM-1 protein expression in iron-stimulated HUVECs by Western blotting. HUVECs were incubated with or without 50 µg/ml IV iron sucrose or IV FCM and 2 mM NAC for 24hr. TNF-α (120U/ml, 6hr) was used as a positive control. **(A):** show protein band of 95 kDa representing VCAM-1 and (S) represents iron sucrose. **(B):** data (mean ± SEM) are shown as a bar graph of densitometry data from 3 different donors (n=3). *p<0.05, **p<0.01 and ***p<0.01 compared with non-treated cells. $^{\#\#}p$<0.01 for IV iron sucrose or IV FCM and NAC together at 24hr compared with IV iron sucrose or IV FCM alone at 24hr.

5.4. DISCUSSION

Atherosclerosis is an inflammatory vascular disease, which presents frequently in patients with CKD (Amann *et al*., 2004). EC activation has been proposed to be one of the initiating events of atherosclerosis and it is characterized by the induction of several intracellular signalling pathways leading to up-regulation of several proteins including CAMs, which will be expressed and mobilized to the activated EC surface (Lusis, 2000; Galkina and Ley, 2009). During EC activation, ICAM-1 and VCAM-1 are up-regulated, and play an important role in early stages of atherosclerosis by enabling the circulating mononuclear cells (MNCs) to bind to ECs and migrate into the sub-endothelial space (Badimon *et al*., 2012).

In this study, to investigate whether IV iron preparations induce EC activation, we have looked into the effect of the two commercially and routinely used forms of IV iron preparations from earlier chapters (IV iron sucrose or IV FCM) on ICAM-1 and VCAM-1 expression in HUVEC. Firstly, an established RT-PCR technique was used in this study and we observed that the level of gene expression of both ICAM-1 and VCAM-1 mRNA were up-regulated in HUVEC treated by IV iron sucrose or IV FCM at 6 and 24hr compared to non-treated cells (Figures 5.2A and 5.3A). To quantify the data obtained by RT-PCR in gene expression, qRT-PCR was performed. QRT-PCR data clearly showed that HUVEC treated with IV iron sucrose or IV FCM at 2hr increased ICAM-1 and VCAM-1 mRNA gene expression levels, however this increase was not statistically significant compared with non-treated cells. In contrast, cells treated with IV iron sucrose or IV FCM at 6 and 24hr, showed significant increase in ICAM-1 and VCAM-1 mRNA gene

expression levels compared with non-treated cells (Figures 5.6 and 5.7). Although gene expression level of ICAM-1 and VCAM-1 was up-regulated, it is important to determine if surface expression of protein is altered. Therefore, Western blotting was performed using specific anti-ICAM-1 and anti-VCAM-1 antibodies. ICAM-1 was detectable in non-treated cells in a similar way to published results because it is well known that HUVEC express low level of ICAM-1 under basal conditions (Salas *et al*., 2002). In contrast, no VCAM-1 was detected in our study, in agreement with others that VCAM-1 is not expressed on resting healthy HUVEC surface but expressed only on activated HUVEC (Frank and Lisanti, 2008). HUVEC treated with IV iron sucrose or IV FCM at 6 and 24hr showed significant increase in protein levels of ICAM-1 compared with non-treated cells (Figure 5.8B). In addition, only cells treated with IV iron sucrose at 6 and 24hr or IV FCM at 24hr showed significant increase in protein levels of VCAM-1 compared with non-treated cells (Figure 5.9B). Our data are line with a number of studies which investigated the role of IV iron preparations on adhesion molecule expression in other vascular cell types. A recent study by Kuo *et al* (2014) showed that, HAECs treated with IV iron sucrose at 80 and 160 µg/ml for 5hr, significantly up-regulated ICAM-1 and VCAM-1 protein expression in these cells via the enhancement of NF-kB binding activity. Furthermore, *in vivo* study by Kuo *et al* (2014) who, treated 20 CKD patients at stage 5 (ESRD) with 1000 mg IV iron sucrose showed that, plasma levels of soluble sICAM-1 and sVCAM-1 were significantly up-regulated compared with those who were treated with IV iron compound. In addition, Martin-Malo *et al* (2012) treated primary PBMCs isolated from CKD patients with four IV

iron preparations, ferric gluconate, iron sucrose, iron dextran and IV FCM at a concentration of 200 µg/ml for 2, 8 and 24hr. Their data showed that all compounds caused significant increases in ICAM-1 protein expression. Interestingly, the authors claimed that, there were no significant differences between the four iron preparations in the level of ICAM induced (p<0.05).

Kuo *et al* (2012), treated HAEC *in vitro* with 80 and 160 µg/ml IV ferric chloride hexahydrate for 4hr and measured adhesion molecule expression using Western blot methods. They found that protein level of VCAM-1 was significantly up-regulated by IV ferric chloride hexahydrate at 80 and 160 µg/ml for 4hr, while ICAM-1 protein expression was significantly increased only at 160µg/ml for 4hr. In addition, these authors also showed this was the case *in vivo*, when they treated a group of HD patients with IV ferric chloride hexahydrate at concentration of 400 and 800 mg over 6 months. They found that, plasma level of soluble sICAM-1 and sVCAM-1 were significantly elevated at 800 mg compared with patients who did not receive IV iron treatment, demonstrating sustained endothelial cell activation. Although our study is consistent with the findings of Kuo *and* colleagues study, we did not measure up-regulation at 4hr but levels were significantly up-regulated at 6hr and increased further at 24hr. In addition, the authors showed that ICAM-1 protein expression was not significantly up-regulated by 80 µg/ml IV ferric chloride hexahydrate at 4hr and they did not look at a longer time exposure, so they might have looked too early to see up-regulation of expression of ICAM-1 (Hadad *et al*., 2011). Although, our study used a lower concentration of IV iron sucrose or IV FCM (50 µg/ml), our success in demonstration up-regulation of ICAM-1 and VCAM-1 protein is likely to be due to the time point

chosen in our study. Collectively both our and published observations could be considered as markers of EC activation/dysfunction, which indicate that IV iron preparations could accelerate early atherogenesis through endothelial activation and up-regulation of adhesion molecule expression. In contrast to the data by Kuo's group, one *in vivo* study involving 40 HD patients showed that treating those patients with 50 and 100 mg/kg IV iron sucrose had no significant effect on level of serum sICAM-1 (Garcia-Fernandez *et al*., 2010). Another recent *in vivo* study by Prats *et al* (2013) showed that there was no significant increase in ICAM-1 and VCAM-1 expression compared with baseline after treating 47 CKD patients at stages 3-5 with IV FCM (15 mg/kg). The authors stated that the lack of IV FCM effect on expression level of ICAM-1 and VCAM-1, could be due to IV FCM characteristics itself where there is less possibility, when administrated even at high doses did not release large amounts of ionic iron in the serum. In addition, Prats *and* others also noted that patients who were on lipid lowering treatment have a lower level of VCAM-1, suggesting that these drugs might block the effect of IV FCM on adhesion molecule (Prats *et al*., 2013). However, Prats *and* others study cannot be directly compared to our and other *in vitro* data as they measured the expression of ICAM-1 and VCAM-1 levels at 1hr, 3 weeks and then 3 months after administration of IV FCM. Thus, this period of time measurements are taken can be crucial as they could have missed the increased level of adhesion molecule between 1hr of IV FCM administration and three weeks. Previous clinical and experimental studies showed the protective effect of NAC on reducing ICAM-1 and VCAM-1 adhesion molecule expression in ECs treated with IV iron preparations, iron sucrose,

iron dextran, FCM and ferric chloride hexahydrate (Schmidt *et al*., 1997; Weig and *et al*., 2001; Kuo *et al*., 2012). Our results showed that, NAC significantly reduced protein expression of adhesion molecule ICAM-1 and VCAM-1 in HUVEC treated with IV iron sucrose or IV FCM over 24hr $p<0.01$, as shown in Figure 5.10 A and B. Our data implies that antioxidants such as, NAC may potentially protect against iron-mediated endothelial dysfunction. The mechanisms responsible for NAC's effects are more fully discussed in Chapter 6. The NAC and iron preparation co-incubation data may indicate that, IV iron sucrose or IV FCM induced ROS generation, which in turn might promote endothelial adhesion molecule expression through the activation of NF-kB and subsequently promote early atherogenesis in CKD patients.

Our results of co-treatment of HUVEC with NAC and IV iron preparation are consistent with the two studies by Kuo *and* colleagues, where expression of ICAM-1 and VCAM-1 was significantly attenuated by the co-treatment of ferric chloride hexahydrate-treated HAEC with 10 mM NAC (Kuo *et al*., 2012; Kuo *et al*., 2014). This group needed 10 mM NAC to produce significant effects of IV iron preparation induced on ICAM-1 and VCAM-1 protein expression. However, we did not use such high concentrations as previous shear stress studies in our laboratory showed that, treating HUVEC with 10 mM NAC significantly blocked all interactions between DL-homocysteine-treated HUVEC and neutrophils and reduced interactions to below non-treated control level, which indicates that NAC at such high concentration might have affected the cellular response to stimuli (Alkhoury, 2009).

We carefully chose clinically relevant IV iron concentrations for our studies (Kamanna *et al*., 2012), yet intriguingly, all of the other published studies

have used higher concentrations of IV iron preparations than those used in our study. However, despite the lower concentrations that were used in our study, there was still up-regulation of adhesion molecules, which was significantly attenuated by pre-treatment with NAC. This suggests that treatments with IV iron sucrose or IV FCM gives rise to oxygen radicals. This can lead to EC dysfunction through up-regulation of EC adhesion molecule, ICAM-1 and VCAM-1 which play vital role in the prevalence of atherosclerosis, a major CVD in CKD patients and, thus potentially increase the mortality in these groups.

5.5. CONCLUSION

In conclusion, the results of our *in vitro* experiments provide compelling evidence that IV iron sucrose or IV FCM significantly induce EC activation through both increased mRNA expression and protein up-regulation of ICAM-1 and VCAM-1 adhesion molecules in cultured HUVEC. In addition, our study, showed for the first time, that IV FCM increases mRNA expression and up-regulates ICAM-1 and VCAM-1 protein expression in EC (HUVEC). These findings will result in EC dysfunction and increase the mortality of CKD patients as a result of CVD progression. It was interesting to note that adhesion molecule upregulation by IV FCM was not as high as that by IV iron sucrose. The fact that, treatment with NAC significantly attenuated the effect of IV iron administration on adhesion molecule protein expression emphasises the role of ROS in the endothelial cell damage sustained.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE STUDIES

6.1. GENERAL DISCUSSION AND FUTURE STUDIES

6.1.1. General discussion

In vitro studies have used ECs from a variety of sources to study the mechanisms of EC dysfunction that lie behind atherosclerotic plaque formation (Zhang *et al*., 2001a; Lee *et al*., 2002; Dalal *et al*., 2003; Ferretti *et al*., 2004). In our study HUVECs were chosen for several reasons, initially as these cells represent one easily accessible *in vitro* model principally because of the umbilical cord availability. These cells have played a major role as a model system for the study of EC function and have been used successfully in several studies (Outinen *et al*., 1999; Xu *et al*., 2000; Dong *et al*., 2005; Xu et al., 2011). Although atherosclerotic lesions occur in large arteries and veins are not typically vulnerable to the development of atherosclerosis (VanderLaan *et al*., 2004), venous endothelium plays a key role in inflammation as veins provides an important site of leukocyte recruitment to venule walls (Savoia *et al*., 2011). This results in the up-regulation of adhesion molecule expression (including ICAM-1, VCAM-1). Patients with CKD present with systemic inflammation as measured by elevated levels of inflammation markers including CRP, TNF-α, and interleukin -6 (IL-6), which have been shown to correlate with CV outcome (Dungey *et al*., 2013, Machowska *et al*., 2015).Therefore, inflammation associated with EC dysfunction is considered as CVD risk factors for patients with CKD. Thus, all types of ECs are likely to be activated, leading to leukocyte recruitment into kidney tissue which will further promote chronic inflammation, and making the use of HUVEC in our study relevant in determining the mechanisms of EC activation/damage important in CKD.

Both *in vivo* and *in vitro* studies have been conducted to determine the effect of IV iron preparations on oxidative stress and the role of these compounds on EC dysfunction and subsequent CVD progression and this will be discussed in the context of our data (Rooyakers *et al*., 2002; Kell, 2009; Vaziri, 2012; Kamanna *et al*., 2012). Most published studies investigated the role of IV iron sucrose, because it has been the most commonly used in the clinic (McMahon, 2010; Cançado and Muñoz, 2011; Kayode *et al*., 2012). Therefore, it was one obvious choice for our studies as a benchmark of a routinely used therapeutic. In contrast, little is known about the role of IV FCM on EC dysfunction, because it is considered as a newer clinical agent. Most recent scientific investigations have focused mainly on *in vivo* (patient) study, with most of these studies concluding this drug is safe (Barish *et al*., 2012; Bregman and Goodnough, 2014; Macdougall *et al*., 2014), with data indicating similar effects on EC function measured by serum biomarkers to iron sucrose. However, the research that has been conducted and the relatively short time the agent has been approved in Europe and USA means there is insufficient *in vitro* experimental evidence on the effects of this compound on EC function. Long term administration to CKD patients may lead to acceleration of CVD through EC damage in the kidney. Therefore, our *in vitro* study aimed to directly compare effects of these two commercially available compounds on EC function, particularly to compare their effects on ROS generation and apoptosis in HUVECs as well as studying the potential mechanisms responsible. From early dose response studies using HUVEC and endothelial cell line (EA.hy926), we chose a concentration of 50 µg/ml for both IV iron sucrose and IV FCM, because higher concentration had

adverse effects on EC morphology and apoptosis induced by IV iron sucrose. However, these concentrations are relevant to the clinically measured range of plasma iron concentrations that are achieved in CKD patients after injected with IV iron sucrose or IV FCM as described in section 2.2.2.7. (Van Wyck *et al*., 2004; Kuo *et al*., 2012; Kamanna *et al*., 2012; Praschberger *et al*., 2013). From the experiments carried out in this thesis, our data showed that IV iron sucrose, results in morphological changes in HUVEC within 24hr and cells displayed typical features of apoptosis as these cells shrunk and became star-shaped with sharp outlines compared with non-treated cells from the same donor. In contrast, HUVEC treated with IV FCM showed normal morphology with typical cobblestone appearance compared with non-treated cells. In addition, trypan blue and MTT data following 24hr of treatment showed, IV iron sucrose significantly induced a marked decrease in HUVEC proliferation and inhibition of the survival of the HUVEC when compared with non-treated cells while, IV FCM did not produce EC damage measured by MTT assay at this time point. However, long term treatment with IV iron sucrose or IV FCM, MTT data for 96hr showed that, IV iron sucrose was the more toxic agent inducing further significant reductions in the proliferation of HUVEC by 80% compared with non-treated cells. Interestingly, IV FCM did not kill EC but caused significant delay in HUVEC proliferation rate at 72hr and 96hr compared with non-treated cells. Our results show for the first time that, IV FCM *in vitro* can affect the proliferation rate of EC on long term treatment with repeat dose administration using MTT assay. In order to identify potential therapeutic agents for safe use in CKD patients, it was important to examine exact

mechanism of this IV iron induced EC damage, TUNEL assay and Annexin V-FITC/PI, data demonstrates that IV iron sucrose at 24hr causes significant HUVEC apoptosis compared with non-treated cells. In line with the earlier data IV FCM did not induce HUVEC apoptosis which suggests it may have a safer profile. Our data are in line with the findings of Carlini *et al*., 2006; Martin-Malo *et al*., 2012, who showed that, 100-1000 µg/ml IV iron sucrose significantly increases apoptosis rate in BAEC and PBMC using TUNEL and Annexin V-FITC/PI. In addition, Fell *et al*., (2014) found a significant reduction in monocyte viability treated with 500 µg/ml IV iron sucrose for 3hr versus non-treated cells, which was not the case in cells treated with IV FCM, as there was no significant decrease in the cell viability compared with non-treated cells. Zager *et al* (2002) also showed that IV iron sucrose inhibits HAEC proliferation when these cells were treated with 60 and 1000 µg/ml for 72hr using MTT assay. These groups suggested that impaired mitochondria function was the cause for inhibition of HAEC proliferation by this compound. In addition, another study was done by Carlini *et al* (2006), who agreed with the finding of Zager *et al*, treating BAECs with 500 and 1000 µg/ml IV iron sucrose, showing this compound significantly decreased BAEC proliferation compared with non-treated cells.

It has been shown that IV iron sucrose can cause renal injury due to direct drug toxicity induced by the drug; this injury *per se* can cause the production of oxidative stress and EC damage (Agarwal, 2004). Our data agree with the findings of several *in vitro* studies. Kamanna *et al* (2012) showed that, treatment of HAEC with 100 µg/ml IV iron sucrose had an adverse effect on cell morphology and cells exhibited typical features of apoptosis including

condensed and fragmented nuclei when compared to non-treated cells. They also showed that IV iron sucrose at 24hr significantly decreased cellular viability using MTT assay compared with non-treated cells. These authors suggested that, alteration of ECs morphology could be due to the increased oxidative stress after IV iron being administered. Furthermore, Masuda *et al* (2014) showed that 200 mg/l IV iron sucrose resulted in significant decrease in viability of isolated pancreatic islet cells using tetramethylrhodamine ethyl ester (TMRE) methods, which occurred as a result of increased oxidative stress. Several studies, both on CKD patients and experimental animals have demonstrated the capability of IV iron preparations to cause oxidative stress (Agarwal, 2004; Agarwal, 2006; Anirban *et al*., 2008). In addition, *in vitro* studies have also shown that IV iron preparation such as IV iron sucrose, iron dextran, IV FCM and ferric gluconate and ferric chloride hexahydrate treatment, induced ROS generation in HAEC, PBMC and pancreatic islet cells (Kuo *et al* 2008; Gupta *et al*., 2010; Kuo *et al*., 2012; Martin-Malo *et al*., 2012; Fell *et al*., 2014; Masuda *et al*., 2014).Our results obtained using both NBT and DCFH-DA showed that, treatment with IV iron sucrose or IV FCM induced significant increases in superoxide anion production and total ROS generation in HUVEC when compared with non-treated HUVEC, leading to oxidative stress. Our data are in line with the above mentioned studies, which suggest the increase in ROS can be due to a number of reasons including the molecular weight and stability of the IV iron compounds, which then release more free iron Fe^{2+} that could enter the cells via divalent metal transporter (DMT1) (Sturm *et al*., 2005) or it could be due to IV iron altering the intracellular labile iron pool, leading to increased

oxidative stress by affecting the antioxidant enzymes system for instance, glutathione reductase and catalase (Gupta *et al.*, 2010). After showing the ROS generation and apoptotic effects of IV iron sucrose on HUVEC, the mechanism was next studied. ROS are known to mediate the activation of multiple signalling pathways, including p38 MAPK signalling pathway (Sato *et al*., 2014), and are a known stimulus of p38 MAPK in EC (Usatyuk *et al*., 2003). Many studies have shown that apoptosis can be mediated by p38 MAPK in EC, including BPAEC or EA.hy926 cell line induced by TNF-α (Yue *et al*., 1999; Grethe *et al*., 2004). Therefore, we confirmed by Western blot data that IV iron sucrose or IV FCM each significantly activated p38 MAPK in HUVEC in time dependent manner (peaking at 1 and 2hr). However, phosphorylation of p38 MAPK in HUVEC treated by IV FCM was less significant and not to the same extent as IV iron sucrose-treated HUVEC. Strong evidence provided in the literature suggests a key role for p38 MAPK pathway in induction of the apoptosis by mediating the phosphorylation of Bcl-2 and the translocation of Bax to the mitochondria (De Chiara *et al*., 2006; Kang *et al*., 2008; Park *et al*., 2013), It was important to link the activation of p38 by IV iron sucrose to apoptosis we detected, as IV FCM and iron sucrose both induced p38 but did not both induce apoptosis. To do this we examined Bcl-2 and Bax proteins involved in the regulation of the apoptotic stress pathway (Soria *et al*., 2013). Treatment of HUVEC with IV iron sucrose significantly down-regulated Bcl-2 and upregulated Bax protein expression compared with non-treated cells, confirming the role of these the two proteins in the apoptotic stress pathway. In contrast, IV FCM had no effect on Bcl-2 and Bax expression. These results are in line with Carlini *et*

al., (2006) who found that, BAECs treated with 100 and 1000 µg/ml IV iron sucrose, demonstrated significant down-regulation of Bcl-2 protein expression. However, no change in Bax protein expression was observed. The authors suggested that, the expression of Bax was not changed as both Bcl-2 and Bax are acting independently. In contrast, our data showed that both proteins were affected by IV iron sucrose, in a way which would promote apoptosis confirming that up-regulation of Bax is essential for cell death, either through binding of Bax to Bcl-2 and therefore possibly inactivating it or by creating cytotoxic channels for ions or other molecules in the intracellular membranes where it resides (Zha and Reed, 1997). In addition, cell type that has been used in our study might be more sensitive to IV iron sucrose to those used by these authors.To confirm that p38 MAPK phosphorylation contribute to mitochondria integrity in IV iron sucrose-treated HUVEC through the imbalance between the apoptotic proteins, Bcl-2 and Bax, HUVEC were treated with the p38 MAPK inhibitor, SB203580. On pre-treatment with SB203580 HUVEC showed partially but significantly reduced expression of Bax and increased expression of Bcl-2 protein compared with cell treated with IV iron sucrose alone and the percentage of apoptotic cells was significantly reduced to 19%. These data indicate that the activation of p38 MAPK is a critical link affecting the balance of expression of Bcl-2 and Bax, and their involvement in IV iron sucrose induced apoptosis. However, treatment with IV iron sucrose and SB203580 was not comparable to non-treated cells, which indicates that apoptosis in response to IV iron sucrose treatment in HUVEC is likely to involve either different signalling pathways involved in HUVEC apoptosis or could be through directs effect of the drug

toxicity on HUVECs. This is worthy of further investigation, (see future studies, section 6.2.1). These results are in line with Grethe *et al* (2004); Yu *et al* (2006) who showed that inhibition of p38 MAPK by SB203580 attenuated the apoptosis in EA.hy926 cell line exposed to TNF-α or H_2O_2. Additionally, SB203580 has been shown to upregulate Bcl-2 and down regulate Bax expression in HUVEC exposed to Lipopolysaccharide (Lim *et al*., 2009).

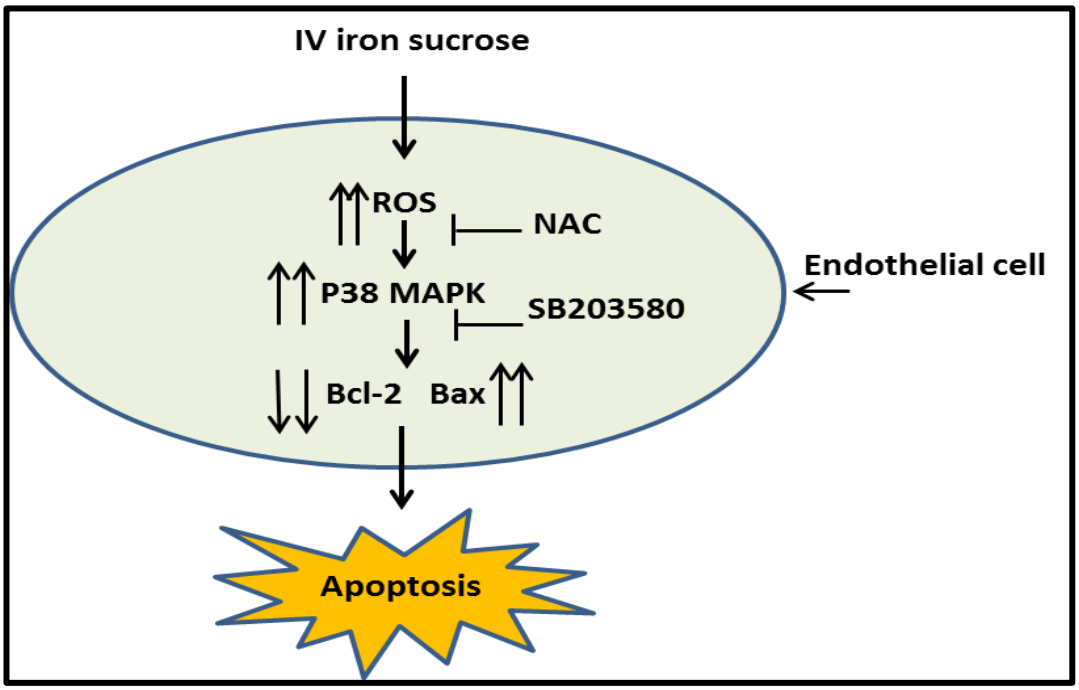


Figure 6.1: Schematic diagram of proposed mechanism of IV iron sucrose lead to EC apoptosis. IV iron sucrose increases ROS generation in HUVEC which can mediate the activation of p38 MAPK, leading to down-regulation of Bcl-2 and up-regulation of Bax, and subsequently cell apoptosis. ROS generation can be prevented by treatment with antioxidant, NAC demonstrating that ROS cause direct activation of p38 MAPK.

While apoptosis is a critical feature important in CVD, EC activation is the key mechanism underlying chronic inflammatory conditions including atherosclerosis, which in turn lead to CVD (Sorensen *et al*., 1994; Gokce *et al.*, 2002; Libby *et al*., 2002), which is the main cause of death in CKD patients (Levey and Eknoyan, 1999). EC activation is characterized by increased vascular permeability and up-regulation of adhesion molecule expression, including ICAM-1, VCAM-1 (Libby *et al*., 2002). Hence, we examined the role of IV iron preparations in EC activation that might play a critical role in atherosclerosis and subsequently CVD progression in CKD patients. Results obtained by both RT-PCR and qRT-PCR showed that, IV iron sucrose or IV FCM induced significant up-regulation of both ICAM-1 and VCAM-1 mRNA compared with non-treated cells. Downstream protein expression was also significantly up-regulated in response to the 2 iron preparations as shown in Figure 6.2. Interestingly our data shows that there is more significant upregulation of adhesion molecule protein expression following treatment with IV iron sucrose, compared to IV FCM. Our data are in line with number of studies that showed that 1000 mg IV iron sucrose or 200 µg/ml IV iron sucrose, iron dextran, ferric gluconate and IV FCM all significantly activated the expression of soluble and surface, ICAM-1 and VCAM-1 protein expression in CKD patients (Martin-Malo *et al*., 2012; Kuo *et al*., 2014). 160 µg/ml IV ferric chloride hexahydrate also significantly elevated the surface expression of VCAM-1 protein and soluble ICAM-1 and VCAM-1 in serum (Kuo *et al*., 2012), of patients with CKD (Frank and Lisanti, 2008). All the above findings could be considered as demonstrating that CKD

patients treated with IV iron preparations demonstrate expression of markers of EC activation/dysfunction.

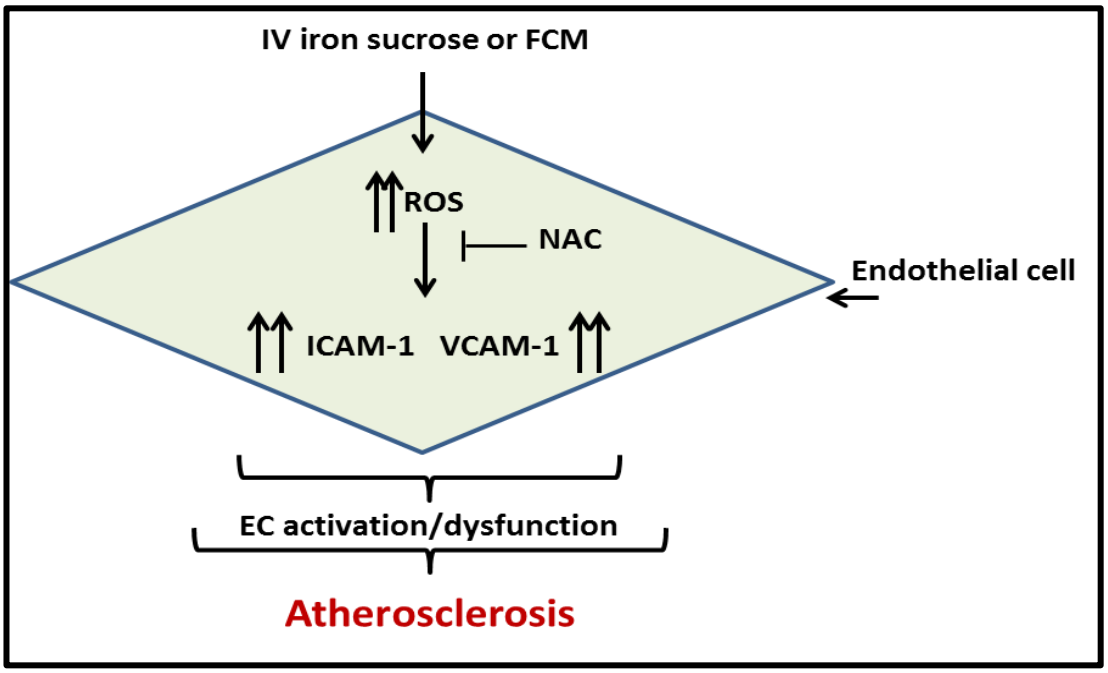


Figure 6.2: Schematic diagram of proposed mechanisms of IV iron preparations result in ROS generation in HUVEC, up-regulation of ICAM-1 and VACM-1, EC activation/dysfunction and therefore atherosclerosis, which can be attenuated by treatment with antioxidant, NAC.

In our studies co-treatment with antioxidant, 2 mM (NAC) significantly attenuated the production of both superoxide anion and ROS generation induced by IV iron sucrose or IV FCM. These results are matched with others (Agarwal *et al.,* 2004; Swarnalatha *et al*., 2010), who found that ROS induction in response to 100 mg of IV iron sucrose and 600 mg NAC treatment resulted in significant reduction of plasma MDA compared to CKD patients without NAC treatment. We hypothesized that EC damage, apoptosis and p38 MAPK activation induced by IV iron preparation are ROS dependent, and might be improved by antioxidant treatments of NAC. The activation of p38 MAPK in response to IV iron preparation treatment in

HUVEC was ROS dependent and our experiments convincingly showed NAC, reduced the phosphorylation of p38 MAPK in HUVEC treated with IV iron sucrose or IV FCM compared with non-treated cells. This confirmed that p38 MAPK activation was ROS dependent, suggesting that p38 MAPK activation can lead to EC apoptosis. These findings are in line with other studies that showed 5 mM NAC and 100 µM H_2O_2 significantly reduced activation of p38 MAPK in BLMVECs compared with H_2O_2-treated cells (Usatyuk *et al*., 2003; Wang and others, 2010). In addition, our data also showed that NAC significantly attenuated the protein expression of ICAM-1 and VCAM-1 in HUVEC treated with IV iron sucrose or IV FCM compared with IV iron sucrose or IV FCM alone, indicating that the up-regulation of gene and increased protein expression of ICAM-1 and VCAM-1 which may facilitate leukocyte adhesion to the vascular endothelium, leading to EC dysfunction and atherosclerosis is ROS dependent. These results are consistent with the two studies by Kuo and colleagues who showed that NAC significantly reduced the expression of proteins ICAM-1 and VCAM-1 in lymphocytes (Kuo *et al*., 2012; Kuo *et al*., 2014). Therefore, the reduction of proliferation rate and EC damage by IV iron preparation would be expected as consequences of increased level of ROS particularly superoxide anion production. Collectively our results suggest that, ROS were responsible for activating the p38 MAPK pathway and downstream pathways inducing apoptosis in HUVEC via the up-regulation of Bax and down-regulation of Bcl-2 in response to IV iron sucrose as well as EC activation or dysfunction.

Our results show for the first time that IV iron sucrose or IV FCM activated p38 MAPK expression in HUVEC can be as a result of ROS induction.

However, IV FCM did not induce HUVEC apoptosis; suggesting the role of additional pathways in the induction of apoptosis alongside p38 MAPK. Pharmacokinetic differences exist between IV iron sucrose and IV FCM such as rapid iron absorption, distribution, metabolism and excretion. In addition, differences in their molecular weight, reactivity and thermodynamic stability, which differ in their carbohydrate ligands leading to passive absorption, saturation of the transport protein (transferrin) and generation of weakly bound NTBI, which can prompt oxidative stress and cellular damage (Geisser and Burckhardt, 2011). We suggest that IV iron sucrose can be toxic and can cause apoptosis in HUVEC via all these differences which are mentioned and discussed in more details in section 1.6.3.1. Although, our data shows additional information on mechanisms by which IV iron sucrose or IV FCM may damage ECs, these effects may not be observed *in vivo*. Future studies are needed to determine the exact mechanism underlying IV iron sucrose-induced EC apoptosis (see future studies, section 6.1.2). Overall, our data in this thesis concludes that low and pharmacologically relevant concentration of both IV iron preparations affect EC function. Importantly IV iron sucrose exerts a greater inhibitory effect on cell proliferation, intracellular ROS generation and superoxide anion production than IV FCM in EC. Additionally, IV iron sucrose produced greater effects on apoptosis and cellular activation by the up-regulation of key EC adhesion molecules, which regulate monocyte recruitment and play a pivotal key role in early atherosclerosis compared with IV FCM. The mechanisms involved in EC activation may be partly due to the increased oxidative stress by both compounds. Furthermore, Cell toxicity of IV iron sucrose occurs through

induction of HUVEC apoptosis and the mechanisms involved in this process may be related to up-regulation and down-regulation of Bax and Bcl-2 protein expression respectively, which shown to be at least partly mediated by the activation of p38 MAPK. This study also highlighted the possibility of antioxidant NAC and p38 MAPK inhibition as protective agents against HUVEC oxidative stress and damage. All these *in vitro* findings suggest a potential novel causative role for therapeutic IV iron preparations particularly IV iron sucrose in EC dysfunction and provide convincing evidence for IV iron preparations in the pathogenesis of atherosclerosis and CV complications in CKD patients, which might result in significant morbidity and mortality in these individuals. What is promising is that this study has shown that IV FCM can be more promising in anaemia treatment for CKD patients than IV iron sucrose as it shown to have a higher efficacy and less toxicity on HUVEC to IV iron sucrose for similar compound concentration and exposure to cells. Thus, IV FCM may have positive clinical consequences such as less oxidative stress and less cell damage/dysfunction. However, further work needs to be done to confirm these promising preliminary findings and clinically it will be important to look at the mean survival time or to compare the percentage of CKD patients treated with IV iron sucrose or IV FCM who die due to CVD. Effects of long terms treatment with either compound on EC dysfunction in CKD patients using physiological techniques such as flow mediated dilatation or measurement of soluble cell adhesion molecules would be beneficial in assessing the comparative effects of these drugs on CV function.

6.1.2. Future studies

Based on findings and discussion in this thesis, additional techniques could be used to further investigate the role and mechanisms of IV iron preparation-induced EC dysfunction/activation and apoptosis, which will eventually result in CVD progression in patients with CKD.

1. Investigate whether human serum from late stage CKD or HD patients, who are on short and long term treatment of IV iron preparation (IV iron sucrose or IV FCM), impair EC function in healthy cultured HUVEC. This is to mimic conditions present in CKD patients as this serum may contain high level of metabolites able to induce oxidative stress, either because of IV iron preparations treatment or by uremic toxins which known for its role in inhibition antioxidant enzymes in CKD (Kumbasar *et al*., 2012). Briefly, whole blood samples can be collected from age and disease stage matched CKD patients who are on short (1-3 months from first dose) or long treatment (6-12 months) with IV iron preparations (IV iron sucrose or IV FCM). Serum samples then can be isolated from whole blood by centrifugation and HUVEC from healthy donors can be incubated with this serum for different time course (2, 6 and 24hr). Subsequently, cell viability, proliferation, production of intracellular ROS, superoxide anion production, cell activation (up regulation of cell adhesion molecules) and apoptosis can be measured *in vitro* using the same methods in this study and whether treatment with NAC can attenuate the effect of IV iron preparations.

2. Since IV iron sucrose or IV FCM up-regulated EC adhesion molecules, *in vitro* flow experiments could also be performed to simulate laminar blood flow through a human blood vessel. Classification of EC: leukocyte interactions (tethering, rolling, fixed and transmigration) will be investigated using Vacuum Flow Chamber Assay (Shishir Shetty *et al*., 2014) to observe the effect of:
   a) The recruitment of leukocytes from healthy individuals on HUVEC which have been pre-treated for different time course (2, 6 and 24hr) with serum from late stages CKD or on dialysis patients who are on either short (1-3 months) or long term (6-12 months) of IV iron sucrose or IV FCM.
   b) The recruitment of leukocytes isolated from blood of CKD patients who are on either short (1-3 months from first dose) or long term (6-12 months) of IV iron sucrose or IV FCM on healthy HUVEC.
   c) The recruitment of leukocytes isolated from blood of CKD patients who are on either short (1-3 months) or long term (6-12 months) of IV iron sucrose or IV FCM on activated HUVEC (which have been pre-treated for time course in a) with serum from the same patient.
   d) Determine roles of antioxidant treatment on leukocyte recruitment in CKD model under flow conditions, and this will be as follow: leukocytes isolated from blood from late stages CKD or on dialysis patients and NAC to determine the effect of ROS from blood cells on EC activation. HUVEC (which have been pre-treated for time course in a) with CKD patient serum and NAC to determine protective effect of antioxidant on EC function.

3. Directly quantify the strength of the p38 MAPK signal following IV iron sucrose or IV FCM treatment by carrying out p38 MAPK kinase assay. Confirmation of importance of the role of p38 MAPK pathway activation in apoptosis of HUVEC following IV iron sucrose treatment by using molecular methods such as using small interfering RNA (siRNA) (Bramsen and Kjems, 2011) to knock down p38 MAP kinase and measure apoptosis compared to cells where p38 MAPK signal is intact.
4. JNK pathway is also known to play a role in apoptosis in some cell types (Dhanasekaran *et al*., 2008). The role of the JNK pathway in HUVEC apoptosis and alterations in intrinsic pathway proteins, Bax and Bcl-2, will be demonstrated through selective inhibitors such as SP600125 using techniques previously described in this thesis (section 4.2.2.1.3 and 4.2.2.1.3), include Western blotting and flow cytometry with Annexin V-FITC/PI. Confirmation of importance of the role of JNK pathway activation in apoptosis of HUVEC following IV iron preparation treatment could also be confirmed using the siRNA technology to knock down JNK pathway and measure apoptosis as previously described for p38 MAPK in 3.
5. From the findings of this study, the significant increase in ROS generation observed in response to IV iron sucrose or IV FCM will be likely to reduce NO bioavailability (Kolluru *et al*., 2012; Hsieh *et al*., 2014), which plays a major role in EC dysfunction and is a contributor to atherosclerosis in CKD patients. Therefore, evaluation of intracellular NO production in HUVEC could be investigated to look at

the role of ROS induced by IV iron compounds in NO production by treating HUVEC with serum of CKD patients for different time course (2, 6 and 24hr) as described above using NO-specific fluorescent dye 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF2-DA) (Li *et al*., 2010) by flow cytometry. The data obtained from this experiment might show a reduction in NO production which is known to enhance expression of adhesion molecules such as VCAM-1 and ICAM-1. This can provide more detailed information about the role of elevated ROS by IV iron preparations in EC activation/dysfunction which will eventually lead to CVD in patients with CKD.

6. The importance of revealing the mechanism responsible for activation of HUVEC and subsequent apoptosis may lead to usage of novel new small molecules which target these pathways as future therapeutics to protect against accelerated vascular disease within CKD patients. In addition to NAC and SB203580, there are other molecules or drugs which can be used to improve EC function for example NO donors include SP/W 5186 and 3672 as they have shown to inhibit EC activation via inhibition of endothelial leukocyte adhesion molecule expressions, ICAM-1 and VCAM-1 in Human saphenous vein endothelial cells (HSVECs) (Pescador *et al.,* 2013). In addition, angiotensin-converting enzyme inhibitors and angiotensin receptor blockers can be other possible drugs as they shown to improve EC function, and thus slow CKD progression in CKD patients (Wu-Wong, 2008).

REFERENCES

Agarwal, R. (2004). Chronic kidney disease is associated with oxidative stress independent of hypertension," Clinical Nephrology *61*, 377–383.

Agarwal, R. (2006). Proinflammatory effects of iron sucrose in chronic kidney disease. Kidney Int *69*, 1259-1263.

Agarwal, R., Nissenson, A. R., Batlle, D., Coyne, D. W., Trout, J. R., Warnock, D. G. (2003). Prevalence, treatment, and control of hypertension in chronic hemodialysis patients in the United States. Am J Med *115*, 291-7.

Agarwal, R., Vasavada, N., Sachs, N. G., Chase, S. (2004). Oxidative stress and renal injury with intravenous iron in patients with chronic kidney disease. Kidney Int *65*, 2279-2289.

Al-Dadah, A., Omran, J., Nusair, M. B., Dellsperger, K. C. (2012). Cardiovascular mortality in dialysis patients. Adv Perit Dial *28*, 56-9.

Alderton, W. K., Cooper, C. E., and Knowles, R. G. (2001). Nitric oxide synthases: structure, function and inhibition. Biochem J *357*, 593-615.

Alkhoury, K. (2009). Mechanisms responsible for homocysteine mediated damage to human endothelial cells. University of Bradford.

Allen, E. M., Matthews, J. B., O'Halloran, D. J., Griffiths, H. R., Chapple, I. L. (2011). Oxidative and inflammatory status in Type 2 patients with periodontitis. J Clin Periodontol *38*, 894–901.

Amann, K., Gross, M. L., Ritz, E. (2004). Pathophysiology underlying accelerated atherogenesis in renal disease: Closing in on the target. J. Am. Soc. Nephrol *15*, 1664–1666.

Amann, K., Tyralla, K., Gross, M. L., Eifert, T., Adamczak, M., Ritz, E. (2003). Special characteristics of atherosclerosis in chronic renal failure. Clin Nephrol *60*, 13–21.

Anderson, G. F., and Chu, E. (2007). Expanding priorities: confronting chronic disease in countries with low income. N Engl J Med *356*, 209–211.

Andrews, N. C. (1999). Disorders of iron metabolism. N Engl J Med *341*, 1986-1995.

Ang, C., and Lumsden, M. A. (2001). Diabetes and the maternal resistance vasculature. Clin Sci *101*, 719-29.

Anirban, G., Kohli, H. S., Jha, V., Gupta, K. L., Sakhuja, V. (2008). The comparative safety of various intravenous iron preparations in chronic kidney disease patients. Ren Fail *30*, 629–638.

Annuk, M., Zilmer, M., Lind , L., Linde, T., Fellstrom, B. (2001). Oxidative stress and endothelial function in chronic renal failure. J Am Soc Nephrol *12*, 2747–2752.

Ardhanari, S., Alpert, M. A., Aggarwal, K. (2014). Cardiovascular Disease in Chronic Kidney Disease: Risk Factors, Pathogenesis, and Prevention. Adv Perit Dial *30*, 40-53.

Arur, S., Uche, U. E., Rezaul, K., Fong, M., Scranton, V., Cowan, A. E., Mohler, W., Han, D. K. (2003). Annexin I is an endogenous ligand that mediates apoptotic cell engulfment. Dev Cell *4*, 587-98.

Ashkenazi, A. (2002). Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat Rev Cancer *2*, 420-430.

Ashor, A. W., Lara, J., Mathers, J. C., Siervo, M. (2014). Effect of vitamin C on endothelial function in health and disease: a systematic review and meta-analysis of randomised controlled trials. Atherosclerosis *235*, 9-20.

Ashraf, M. I., Ebner, M., Wallner, C., Haller, M., Khalid, S., Schwelberger, H., Troppmair, J. (2014). A p38MAPK/MK2 signaling pathway leading to redox stress, cell death and ischemia/reperfusion injury. Cell Communication and Signaling *12*, 6.

Atmaca, G. (2004). Antioxidant effects of sulfur-containing amino acids. Yonsei Med J *45,* 776-88.

Auclair, C., and Voisin, E. (1985). Nitroblue tetrazolium reduction. In: Greewald, R. A. (Eds). CRC Handbook of Methods for Oxygen Radical Research. CRC Press, Boca Raton, FL 123-132.

Augustin, H. G., Kozian, D. H., Johnson, R. C. (1994). Differentiation of endothelial cells: analysis of the constitutive and activated endothelial cell phenotypes. Bioassays *16*, 901-6.

Au-Yeung, K. K., Woo, C. W., Sung, F. L., Yip, J. C. (2004). Hyperhomocysteinemia activates nuclear factor-kappaB in endothelial cells via oxidative stress. Circ Res *94*, 28-36.

Babior, B. M., (2004). NADPH oxidase. Curr. Opin. Immunol *16*, 42-47.

Babior, B. M., Lambeth, J. D., Nauseef, W. (2002). The neutrophil NADPH oxidase. Arch. Biochem. Biophys *397*, 342-344.

Babitt, J. L., and Lin, H. Y. (2012). Mechanisms of anemia in CKD. J Am Soc Nephrol *23*, 1631-4.

Badimon, L., Padró, T., Vilahur, G. (2012). Atherosclerosis, platelets and thrombosis in acute ischaemic heart disease. Eur Heart J Acute Cardiovasc Care *1*, 60-74.

Bagshaw, S. M., Cruz, D. N., Aspromonte, N., Daliento, L., Ronco, F., Sheinfeld, G., Anker, S. D. (2010). Epidemiology of cardio-renal syndromes: workgroup statements from the 7th ADQI Consensus Conference. Nephrol Dial Transplant *25*, 1406-16.

Bailie, G. R., Clark, J. A., Lane, C. E., Lane, P. L. (2005). Hypersensitivity reactions and deaths associated with intravenous iron preparations. Nephrol Dial Transplant *20*, 1443-1449.

Bakhshi, A., Wright, J. J., Graninger, W., Seto, M., Owens, J., Cossman, J., Jensen, J. P., Goldman, P., Korsmeyer, S. J. (1987). Mechanism of the t (14; 18) chromosomal translocation: structural analysis of both derivative 14 and 18 reciprocal partners. ProcNatlAcadSci USA *84*, 2396-2400.

Banach, M., and Rysz, J. (2010). Current problems in hypertension andnephrology. Expert Opin Pharmacother *11*, 2575-2578.

Banh, S., and Hales, B. F. (2013). Hydroxyurea Exposure Triggers Tissue-Specific Activation of p38 Mitogen-Activated Protein Kinase Signaling and the DNA Damage Response in Organogenesis-Stage Mouse Embryos. Toxicological Sciences *133*, 298-308.

Barish, C. F., Koch, T., Butcher, A., Morris, D., Bregman, D. B. (2012). Safety and efficacy of intravenous ferric carboxymaltose (750 mg) in the treatment of iron deficiency anemia: two randomized, controlled trials. Anemia *2012*, 2012-172104.

Barr, R.K., and Bogoyevitch, M. A. (2001). The c-Jun N-terminal protein kinase family of mitogen-activated protein kinases (JNK MAPKs). Int J of Biochem *33*, 1047-1063.

Baylis, C. (2008). "Nitric oxide deficiency in chronic kidney disease," American Journal of Physiology *294*, 1-9.

BelAiba, R. S., Djordjevic, T., Petry, A., Diemer, K., Bonello, S., Banfi, B. (2007). NOX5 variants are functionally active in endothelial cells. Free Radic. Biol. Med *42*, 446-459.

Belle, E. V., Bauters, C., Asahara, T., Isner, J. M. (1998). Endothelial re-growth after arterial injury: from vascular repair to therapeutics. Cardiovasc Res *38*, 54-68.

Benedetto, F. A., Tripepi, G., Mallamaci, F., Zoccali, C. (2008). Rate of Atherosclerotic Plaque Formation Predicts Cardiovascular Events in ESRD. Journal of the American Society of Nephrology : JASN *19*, 757-763.

Besarab, A. (1999). Iron and cardiac disease in the end-stage renal disease setting. Am J Kidney Dis *34*, 18-24.

Besarab, A., Amin, N., Ahsan, M., Vogel, S. E., Zazuwa, G., Frinak, S., Zazra, J. J., Anandan, J. V., Gupta, A. (2000). Optimization of epoetin therapy with intravenous iron therapy in hemodialysis patients. J Am SocNephrol *11*, 530-538.

Beshara, S., Lundquist, H., Sundin, J. (1999). Kinetic analysis of 52 Fe-labelled iron (III) hydroxidesucrose complex following bolus administration using positron emission tomography. Br J Hematol *104*, 288-295.

Beshara, S., Sörensen, J., Lubberink, M., Tolmachev, V., Långström, B., Antoni, G., Danielson, B. G., Lundqvist, H. (2003). Pharmacokinetics and red cell utilization of ^{52}Fe/^{59}Fe-labelled iron polymaltose in anaemic patients using positron emission tomography. Br J Haematol *120,* 853-9.

Bevilacqua, M. P., Nelson, R. M., and Mannore, G. (1994). Endothelial-leukocyte adhesion molecule in human disease. Annu Rev Med *45*, 361-78.

Bhandari, S. (2011). Beyond efficacy and safety—the need for convenient and cost-effective iron therapy in health care. Clinical Kidney Journal *4*, 14-19.

Biniaz, V., Sadeghi Shermeh, M., Ebadi, A., Tayebi, A., Einollahi, B. (2014). Effect of Vitamin C Supplementation on C-reactive Protein Levels in Patients Undergoing Hemodialysis: A Randomized, Double Blind, Placebo-Controlled Study. Nephro-Urology Monthly *6*, 13351.

Bishu, K., and Agarwal, R. (2006). Acute injury with intravenous iron and concerns regarding long-term safety. Clin J Am Soc Nephrol *1*, 19-23.

Bleyer, A. J., Shemanski, L. R., Burke, G. L., Hansen, K. J., Appel, R.G. (2000). Tobacco, hypertension, and vascular disease: risk factors for renal functional decline in an older population. Kidney Int *57*, 2072-2079.

Boldt, D. H. (1999). New perspectives on iron: an introduction. Am J Med Sc *318,* 207-12.

Bonetti, P. O., Lerman, L. O., Lerman, A. (2003). Endothelial dysfunction: a marker of atherosclerotic risk. ArteriosclerThrombVascBiol *23*, 168-175.

Bonomini, M., Reale, M., Santarelli, P., Stuard, S., Settefrati, N., Albertazzi, A. (1998). Serum levels of soluble adhesion molecules in chronic renal failure and dialysis patients. Nephron *79*, 399-407.

Bottomley, S. S., May, B. K., Cox, T. C., Cotter, P. D., Bishop, D. F. (1995). Molecular defects of erythroid 5-aminolevulinate synthase in X-linked sideroblastic anemia. J Bioenerg Biomembr *27,* 161-8.

Bramsen, J. B., and Kjems, J. (2011). Chemical modification of small interfering RNA. Methods Mol Biol *721,* 77-103.

Braunersreuther, V., Mach, F., Steffens, S. (2007). The specific role of chemokines in atherosclerosis. Thromb Haemost *97,* 714-721.

Bregman, D. B., and Goodnough, L. T. (2014). Experience with intravenous ferric carboxymaltose in patients with iron deficiency anemia. Therapeutic Advances in Hematology *5*, 48-60.

Brevetti, G., Schiano, V., Chiariello, M. (2008). EC dysfunction: a key to the pathophysiology and natural history of peripheral arterial disease? Atherosclerosis *197*, 1-11.

Brissot, P., Ropert, M., Le Lan, C., Loréal, O. (2012). Non-transferrin bound iron: a key role in iron overload and iron toxicity. Biochim Biophys Acta *1820*, 403-410.

Brooks, A. R., Lelkes, P. I., Rubanyi, G. M. (2002). Gene expression profiling of human aortic endothelial cells exposed to disturbed flow and steady laminar flow. Physiol Genomics *9*, 27-41.

Brunelle, J. K., and Letai, A. (2009). Control of mitochondrial apoptosis by the Bcl-2 family. J Cell Sci *122*, 437- 441.

Burkitt, M. J and Wardman, P. (2001). Cytochrome c is a potent catalyst of dichlorofluorescein oxidation: implications for the role of reactive oxygen species in apoptosis. Biochem.Biophys. Res. Commun *282*, 329–333.

Burney, S., Caulfield, J. L., Niles, J. C., Wishnok, J. S., Tannenbaum, S. R. (1999). The chemistry of DNA damage from nitric oxide and peroxynitite. Mutation Research/Fundamental and Molecular Mechanism of Mutagenesis *424*, 37-49.

Cai, H., and Harrison, D. G. (2000). EC dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res 87, 840-844.

Cai, L., Koropatnick, J., Cherian, M. G. (2001). Roles of vitamin C in radiation-induced DNA damage in presence and absence of copper. Chem Biol Interact *137*, 75-88.

Callsen, D., and Brune, B. (1999). Role of mitogen-activated protein kinases in S-nitrosoglutathione-induced macrophage apoptosis.Biochemistry *38*, 2279–2286.

Cançado, R. D., and Muñoz, M. (2011). Intravenous iron therapy: how far have we come? Rev Bras Hematol Hemoter *33*, 461-9.

Cargnello, M., and Roux, P. P. (2011). Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. Microbiol Mol Biol Rev *75*, 50–83.

Carlini, R. G., Alonzo, E., Bellorin-Font, E., Weisinger, J. R. (2006). Apoptotic stress pathway activation mediated by iron on endothelial cells in vitro. Nephrol Dial Transplant *21*, 3055-61.

Cavieres, V., Valdes, K., Moreno, B., Moore-Carrasco, R., Gonzalez, D. R. (2014). Vascular hypercontractility and EC dysfunction before development of atherosclerosis in moderate dyslipidemia: role for nitric oxide and interleukin-6. American Journal of Cardiovascular Disease *4*, 114-122.

Cerutti, P. A. (1985). Prooxidant States and Cancer. Science *227*, 375-381.

Chamoun, F., Burne, M., O'Donnell, M., Rabb, H. (2000). Pathophysiologic role of selectins and their ligands in ischemia reperfusion injury. Front Biosci *5*, 103-109.

Cheesman, K. H., and Salte, T. F. (1993). An introduction to free radical biochemistry, Br. Med. Bull *49*, 481-493.

Chen, J. W., Lin, F. Y., Chen, Y. H., Wu, T. C, Chen, Y. L., Lin, S. J. (2004). Carvedilol inhibits tumor necrosis factor-alpha-induced endothelial transcription factor activation,

adhesion molecule expression, and adhesiveness to human mononuclear cells. Arterioscler Thromb Vasc Biol *24*, 2075-2081.

Cheng, Z., and Li, Y. (2007). What is responsible for the initiating chemistry of ironmediated lipid peroxidation: an update. Chem. Rev *107*, 748–766.

Chertow, G. M., Mason, P. D., Vaage-Nilsen O., Ahlmen, J. (2006). Update on adverse drug events associated with parenteral iron. Nephrol Dial Transplant *21*, 378-82.

Chipuk, J. E., and Green, D. R. (2008). How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? Trends Cell Biol *18*, 157–164.

Chonchol, M., Whittle, J., Desbien, A. (2008). Chronic kidney disease is associated with angiographic coronary artery disease. Am J Nephrol *28*, 354-360.

Chrysohoou, C., Pitsavos, C., Panagiotakos, D. B., Skoumas, J., Stefanadis, C. (2004). Association between prehypertension status and inflammatory markers related to atherosclerotic disease: the ATTICA Study. Am J Hypertens *17*, 568-573.

Cines, D. B., Pollak, E. S., Buck, C. A., Loscalzo, J., Zimmerman, G. A., McEver, R. P., Pober, J. S., Wick, T. M., Konkle, B. A., Schwartz, B. S. (1998). Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood *91*, 3527-3561.

Coen, G., Manni, M., Mantella, D., Pierantozzi, A., Balducci, A., Condò, S., DiGiulio, S., Yancovic, L., Lippi, B., Manca, S., Morosetti, M., Pellegrino, L., Simonetti, G., Gallucci, M. T., Splendiani, G. (2007). Are PTH serum levels predictive of coronary calcifications in haemodialysis patients? Nephrol Dial TransplantNov *22*, 3262-7.

Cohen, G. M. (1997). Caspases: the executioners of apoptosis. Biochem J *326*, 1–16.

Collado, S., Coll, E., Deulofeu, R., Guerrero, L., Pons, M. (2010). Prevalence of cardiovascular disease in uraemia and relevance of cardiovascular risk factors. Nefrologia *30*, 342-8.

Collins, A. J., Li, S., Gilbertson, D. T., Liu, J., Chen, S. C., Herzog, C. A. (2003). Chronic kidney disease and cardiovascular disease in the Medicare population. Kidney IntSuppl *87*, 24-31.

Colombo, P. C., Ganda, A., Lin, J., Onat, D., Harxhi, A., Iyasere, J. E., Uriel, N., Cotter, G. (2012). Inflammatory activation: cardiac, renal, and cardio-renal interactions in patients with the cardiorenal syndrome. Heart Fail Rev *17*, 177-90.

Conrad, M. E., Umbret, J. N., Moore, E. G. (1999). Iron absorption and transport. Am. J. Med. Sci *318*, 213-229.

Cook-Mills, J. M., Marchese, M. E., Abdala-Valencia, H. (2011). Vascular cell adhesion molecule-1 expression and signaling during disease: regulation by reactive oxygen species and antioxidants. Antioxid Redox Signal *15*, 1607-38.

Coollins, A. J., Foley, R. N., Herzog, C. (2009). United States Renal Data System 2008 Annual Data Report. Am J Kidney Dis *53*, 1–374.

Coombes, J. S., and Fassett, R. G. (2012). Antioxidant therapy in hemodialysis patients: a systematic review. Kidney Int *81*, 233-46.

Coresh, J., Astor, B. C., Greene, T., Eknoyan, G., Levey, A. (2003). "Prevalence of chronic kidney disease and decreased kidney function in the adult US population: Third National Health and Nutrition Survey", Am. J. Kidney Dis *41*, 1-12.

Coresh, J., Selvin, E., Stevens, L. A. (2007). Prevalence of chronic kidney disease in the United States. JAMA *298*, 2038-2047.

Coresh, J., Wei, G. L., McQuillan, G., Brancati, F. L., Levey, A. S., Jones, C., Klag, M. J. (2001). Prevalence of high blood pressure and elevated serum creatinine level in the United States: findings from the third National Health and Nutrition Examination Survey (1988–1994). Arch Intern Med *161*, 1207-1216.

Coronado, B. E., Bu, D., Levey, A. S., Meyer, K. B. (1998). Controlling the Epidemic of Cardiovascular disease in chronic renal disease: What do we know? What do we need to learn? Where do we go from here? The Process and Bibliography. Am J Kidney Dis *32*, 14-55.

Costa-Hong, V., Bortolotto, L. A., Jorgetti, V., Consolim-Colombo, F., Krieger, E. M., Lima, J. J. (2009). Oxidative stress and EC dysfunction in chronic kidney disease. Arq Bras Cardiol. 92, 381-6.

Couser, W. G., Remuzzi, G., Mendis, S., Tonelli, M. (2011). The contribution of chronic kidney disease to the global burden of major noncommunicable diseases. Kidney Int *80*, 1258-70.

Cross, J. (2002). Endothelial cell dysfunction in uraemia.Blood Purif *20*, 459-61.

Cross, J. M., Donald, A. E., Nuttall, S. L., Deanfield, J. E., Woolfson, R. G., Macallister, R. J. (2003). Vitamin C improves resistance but not conduit artery endothelial function in patients with chronic renal failure. Kidney Int *63*, 1433-42.

Cuenda, A., and Rousseau, S. (2007). P38 MAP-kinases pathway regulation, function and role in human diseases. Biochim Biophys Acta *1773*, 1358-75.

Dalal, S., Parkin, S. M., Homer-Vanniasinkam, S., Nicolaou, A. (2003). Effect of homocysteine on cytokine production by human endothelial cells and monocytes. Ann ClinBiochem *40*, 534-541.

Danielson, B. G. (2004). Structure, chemistry, and pharmacokinetics of intravenous iron agents. J Am Soc Nephrol *15*, 93-8.

Darzynkiewicz, Z., Galkowski, D., Zhao, H. (2008). Analysis of apoptosis by cytometry using TUNEL assay. Methods *44*, 250-4.

Davidson, S. M., and Duchen, M. R. (2007). Endothelial mitochondria: contributing to vascular function and disease. Circ Res *100*, 1128-1141.

Davignon, J., and Ganz, P. (2004). Role of endothelial dysfunction in atherosclerosis. Circulation *109*, III27-32.

Dayem, A. A., Choi, H. Y., Kim, J. H., Cho, S. G. (2010). Role of oxidative stress in stem, cancer, and cancer stem cells. Cancers Basel *2*, 859-84.

De Chiara, G., Marcocci, M. E., Torcia, M., Lucibello, M., Rosini, P., Bonini, P., Higashimoto, Y., Damonte, G., Armirotti, A., Amodei, S., Palamara, A. T., Russo, T., Garaci, E., Cozzolino, F. (2006). Bcl-2 Phosphorylation by p38 MAPK: identification of target sites and biologic consequences.J BiolChem *281*, 21353-61.

De Chiara, G., Marcocci, M. E., Torcia, M., Lucibello, M., Rosini, P., Bonini, P. (2006). Bcl-2 Phosphorylation by p38 MAPK: identification of target sites and biologic consequences. J Biol Chem *281,* 21353-61.

Deem, T. L., Abdala-Valencia, H., Cook-Mills, J. M. (2007). VCAM-1 activation of endothelial cell protein tyrosine phosphatase 1B. J Immunol *178*, 3865-73.

Degterev, A., Boyce, M., Yuan, J. A. (2003). A decade of caspases. Oncogene *22*, 8543-8567.

Deicher, R., and Horl, W. H. (2002). Intravenous iron: the iatrogenic kick to lose control over oxygen? Kidney Blood Press. Res *25*, 284-288.

Dekkers, J. C., van Doornen, L. J., Kemper, H. C. (1996). The role of antioxidant vitamins and enzymes in the prevention of exercise- induced muscle damage. Sports Med *21*, 213-38.

DeLeo, F. R., Renee, J., McCormick, S., Nakamura, M., Apicella, M., Weiss, J. P., Nauseef, W. M. (1998). Neutrophils exposed to bacterial lipopolysaccharide upregulate NADPH oxidase assembly. J Clin Invest *101*, 455-463.

Derijard, B., Raingeaud, J., Barrett, T., Wu, I. H., Han, J., Ulevitch, R. J. and Davis, R.J. (1995). Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science *267*, 682-685.

Dessì, M., Noce, A., Bertucci, P., Manca, di Villahermosa, S., Zenobi, R., Castagnola, V., Addessi, E., Di Daniele, N. (2013). Atherosclerosis, dyslipidemia, and inflammation: the significant role of polyunsaturated Fatty acids. ISRN Inflamm 191823.

Dhanasekaran, D. N., and Reddy, E. P. (2008). JNK Signaling in Apoptosis. Oncogene 27, 6245-6251.

Dharawhat, A., Golderberg, T., Alam, A., Atat, Ali., Moore Alexis., McFarlane, S. I. (2009). Cardiovascular disease associated with anemia in diabetic patients with chronic kidney disease. International Diabetes Monitor *21*, 171-178.

Dhaun, N., and Webb, D. J. (2013). Novel therapeutic approaches to chronic kidney disease. Br J Clin Pharmacol *76*, 491-4.

Di Angelantonio, E., Chowdhury, R., Sarwar, N., Aspelund, T., Danesh, J., Gudnason, V. (2010). Chronic kidney disease and risk of major cardiovascular disease and non-vascular mortality: prospective population based cohort study. BMJ *341*, 4986.

DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., Karin, M. (1997). A cytokineresponsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature *388*, 548-554.

Diepeveen, S. H., Verhoeven, G. H., van der Palen, J., Dikkeschei, B. L., van Tits, L. J., Kolsters, G., Offerman, J. J., Bilo, H. J., Stalenhoef, A. F. (2004). Oxidative stress in patients with end-stage renal disease prior to the start of renal replacement therapy. Nephron Clin Pract *98*, 3-7.

Dillon, R., Momoh, I., Francis, Y., Cameron, L., Harrison, C. N., Radia, D. (2012). Comparative efficacy of three forms of parenteral iron.J Blood Transfus *2012*, 473514.

Dimmeler, S., and Zeiher, A. M. (2000). Reactive oxygen species and vascular cell apoptosis in response to angiotensin II and pro-atherosclerotic factors. Regul Pept *90*, 19-25.

Dimmeler, S., Hermann, C., Zeiher, A. M. (2000). Apoptosis of endothelial cells. Contribution to the pathophysiology of atherosclerosis? Eur Cytokine Netw *9*, 697-8.

Dimmeler, S., Rippmann, V., Weiland, U., Haendeler, J., Zeiher, A. M. (1997). Angiotensin II induces apoptosis of human endothelial cells. Protective effect of nitric oxide. Circ Res *81*,970-976.

Ding, H., Aljofan, M., Triggle, C. R. (2007). Oxidative stress and increased eNOS and NADPH oxidase expression in mouse microvessel endothelial cells. J Cell Physiol *212*, 682-9.

Dong, F., Zhang, X., Li, S. Y., Zhang, Z., Ren, Q., Culver, B., Ren, J. (2005). Possible involvement of NADPH oxidase and JNK in homocysteine-induced oxidative stress and apoptosis in human umbilical vein endothelial cells.CardiovascToxicol *5*, 9-20.

Drey, N., Roderick, P., Mullee, M., Rogerson, M. A. (2003). Population-based study of the incidence and outcomes of diagnosed chronic kidney disease. Am J Kidney Dis *42*, 677-84.

Dröge, W. (2002). Free radicals in the physiological control of cell function. Physiol. Rev *82*, 47-95.

Drüeke, T., Witko-Sarsat, V., Massy, Z., Descamps-Latscha, B., Guerin, A. P., Marchais, S. J., Gausson, V., London, G. M. (2002). Iron therapy, advanced oxidation protein products, and carotid artery intima-media thickness in end-stage renal disease. Circulation *106*, 2212-2217.

Duffy, S. J., Biegelsen, E. S., Holbrook, M., Russell, J. D., Gokce, N., Keaney, J. F. Jr., Vita, J. A. (2001). Iron chelation improves endothelial function in patients with coronary artery disease.Circulation *103*, 2799-804.

Dungey, M., Hull, K. L., Smith, A. C., Burton, J. O., Bishop, N. C. (2013). Inflammatory factors and exercise in chronic kidney disease. Int J Endocrinol 2013, 569831.

Edgell, C. J. S., Mcdonald, C. C., Graham, J. B. (1983). Permanent cell line expressing human factor VIII-related antigen established by hybridisation. Proc. Natl. Acad. Sci. U.S.A *80*, 3734-3737.

Eknoyan, G., Lamiere, N., Barsoum, R. (2004). The burden of kidney disease: Imporoving global outcomes. Kidney Int *66*, 1310-1314.

Elahi, M. M., Kong, Y. X., Matata, B. M. (2009). Oxidative stress as a mediator of cardiovascular disease. Oxidative Medicine and Cellular Longevity *2*, 259-269.

Eligini, S., Barbieri, S. S., Cavalca, V., Camera, M., Brambilla, M., De Franceschi, M., Tremoli, E., Colli, S. (2005). Diversity and similarity in signaling events leading to rapid Cox-2 induction by tumor necrosis factor-alpha and phorbol ester in human endothelial cells. Cardiovascular Research *65*, 683–693.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. Toxicol Pathol *35*, 495-516.

Eschbach, J. W., and Adamson, J. W. (1985). Anaemia of end-stage renal disease (ESRD). Kidney Int *28*, 1-5.

Eschbach, J. W., Cook, J. D., Scribner, B. H., Finch, C. A. (1977). Iron balance in hemodialysis patients. Ann Intern Med *87*, 710-713.

Esper, R. J., Nordaby, R. A., Vilarino, J. O., Paragano, A., Cacharron, J. L., Machado, R. A. (2006). EC dysfunction: a comprehensive appraisal. Cardiovasc Diabetol *5*, 4.

Falk, E. (2006). Pathogenesis of Atherosclerosis. Journal of the American College of Cardiology *47*, 7-12.

Farley, N., Pedraza-Alva, G., Serrano-Gomez, D., Nagaleekar, V., Aronshtam, A., Krahl, T., Thornton, T., Rincón, M. (2006). P38 mitogen-activated protein kinase mediates the Fas-induced mitochondrial death pathway in CD8+ T cells. Mol Cell Biol *26*, 2118-29.

Feldman, L., Shani, M., Sinuani, I., Beberashvili, I., Weissgarten, J. (2012). N-acetylcysteine may improve residual renal function in hemodialysis patients: a pilot study. Hemodial Int *16*, 512-6.

Félétou, M., and Vanhoutte, P. M. (2006). “EC dysfunction: a multifaceted disorder,” American Journal of Physiology *291*, 985-1002.

Fell, L. H., Zawada, A. M., Rogacev, K. S., Seiler, S., Fliser, D., Heine, G. H. (2014). Distinct immunologic effects of different intravenous iron preparations on monocytes.Nephrol Dial Transplant *29*, 809-22.

Feng, J., Damrauer, S. M., Lee, M., Sellke, F. W., Ferran, C., Abid, M. R. (2010). Endothelium-dependent coronary vasodilatation requires NADPH oxidase-derived reactive oxygen species. Arterioscler Thromb Vasc Biol *30*, 1703-10.

Ferguson, M. A., and Waikar, S. S. (2012). Established and emerging markers of kidney function. Clin Chem *58*, 680-9.

Ferretti, G., Bacchetti, T., Moroni, C., Vignini, A., Nanetti, L., and Curatola, G. (2004). Effect of homocysteinylation of low density lipoproteins on lipid peroxidation of human endothelial cells. J Cell Biochem *92*, 351-360.

Fishbane, S., Pollack, S., Feldman, H. I., Joffe, M. M. (2009). Iron indices in chronic kidney disease in the National Health and Nutritional Examination Survey 1988–2004. Clin J Am Soc Nephrol *4*, 57-61.

Fliser, D., Wiecek, A., Suleymanlar, G., Ortiz, A., Massy, Z., Lindholm, B., Martinez-Castelao, A., Agarwal, R. (2011). The dysfunctional endothelium in CKD and in cardiovascular disease: mapping the origin(s) of cardiovascular problems in CKD and of kidney disease in cardiovascular conditions for a research agenda. Kidney International Supplements *1*, 6–9.

Flora, S. J. (2009). Structural, chemical and biological aspects of antioxidants for strategies against metal and metalloid exposure. Oxidative Medicine and Cellular Longevity *2*, 191-206.

Foley, R. N., Murray, A. M., Li, S., Herzog, C. A., McBean, A. M., Eggers, P. W. (2005). Chronic kidney disease and the risk for cardiovascular disease, renal replacement, and death in the United States Medicare population, 1998 to 1999. J Am Soc Nephrol *16*, 489–95.

Foley, R. N., Parfrey, P. S., Sarnak., M. J. (1998). Epidemiologym of cardiovascular disease in chronic renal disease. J. Am. Soc. Nephrol *9*, 16-23.

Folkes, L. K., Candeias, L. P., Wardman, P. (1995). Kinetics and mechanisms of hypochlorous acid reactions. Arch Biochem Biophys *323*, 120-126.

Foncea, R., Carvajal, C., Almarza, C., Leighton, F. (2000). Endothelial cell oxidative stress and signal transduction. Biol Res *33*, 89-96.

Forkink, M., Smeitink, J. A., Brock, R., Willems, P. H., Koopman, W. J. (2010). Detection and manipulation of mitochondrial reactive oxygen species in mammalian cells. Biochim Biophys Acta *1797*, 1034-44.

Förstermann, U. (2008). Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies Nat Clin Pract Cardiovasc *5*, 338-349.

Förstermann, U., and Li, H. (2011). Therapeutic effect of enhancing endothelial nitric oxide synthase (eNOS) expression and preventing eNOS uncoupling. Br J Pharmacol *164*, 213-23.

Förstermann, U., and Münzel, T. (2006). Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation *113*, 1708-14.

Frank, P. G., and Lisanti, M. P. (2008). ICAM-1: role in inflammation and in the regulation of vascular permeability. Am J Physiol Heart Circ Physiol *295*, 926-927.

Fridovich, I. (1983). Superoxide radical: an endogenous toxicant, Annu. Rev. Pharmacol. Toxicol *23*, 239-257.

Fulda, S., and Debatin, K. M. (2006). Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene *25*, 4798-811.

Fuster, V., Fayad, Z. A., Badimon, J. J. (1999). Acute coronary syndromes: biology. The Lancet *353*, 5-9.

Galkina, E., and Ley, K. (2009). Immune and inflammatory mechanisms of atherosclerosis (*).Annu Rev Immunol *27*, 165-197.

Galley, H. F., and Webster N. R. (2004). Physiology of the endothelium. BRJA *93*, 105-13.

Ganong, W. F. (2005). Review of Medical physiology. McGraw Hill, 547-564.

Gansevoort, R. T., Correa-Rotter, R., Hemmelgarn, B. R., Jafar, T. H., Heerspink, H. J., Mann, J. F. (2013). Chronic kidney disease and cardiovascular risk:epidemiology, mechanisms, and prevention. Lancet *382*, 339-52.

Ganz, T. (2005). Cellular iron: ferroportin is the only way out. Cell Metab *1*, 155–157.

Garcia-Fernandez, N., Echeverria, A., Sanchez-Ibarrola, A., Antonio Paramo, J., Coma-Canella, I. (2010). Randomized clinical trial on acute effects of i. v. iron sucrose during haemodialysis. Nephrology *15*, 178.

Garneata, L. (2008). Intravenous iron, inflammation, and oxidative stress: is iron a friend or an enemy of uraemic patients? J Ren Nutr *18*, 40-5.

Gavrieli, Y., Sherman, Y., Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol *119*, 493-501.

Geisser, P., and Burckhardt, S. (2011). The pharmacokinetics and pharmacodynamics of iron preparations. Pharmaceutics *3,* 12-33.

Geiszt, M. (2006). NADPH oxidases: new kids on the block. Cardiovasc Res *71,* 289-99.

Ghatan, S., Larner, S., Kinoshita, Y., Hetman, M., Patel, L., Xia, Z., Youle, R. J., Morrison, R. S. (2000). P38 MAP kinase mediates bax translocation in nitric oxide-induced apoptosis in neurons. The Journal of cell biology *150*, 335-347.

Gisbert, J. P., Gomollón, F. (2008). Common misconceptions in the diagnosis and management of anaemia in inflammatory bowel disease. Am J Gastroenterol *103*, 1299-1307.

Go, A. S., Chertow G., Dongjie, F., McCulloch, C. E., Hsu, C. Y. (2004). Chronic kidney disease and risks of death, cardiovascular events and hospitalizations. N Engl J Med 351, 1296–1305.

Gogvadze, V., Orrenius, S., Zhivotovsky, B. (2006). Multiple pathways of cytochrome c release from mitochondria in apoptosis. Biochim Biophys Acta *1757*, 639-47.

Gokce, N., Keaney, J. F., Jr., Hunter, L. M., Watkins, M. T., Menzoian, J. O., Vita, J. A. (2002). Risk stratification for postoperative cardiovascular events via noninvasive assessment of endothelial function: a prospective study. Circulation *105*, 1567–72.

Gold, R., Schmied, M., Giegerich, G., Breitschopf, H., Hartung, H. P., Toyka, K. V., Lassmann, H. (1994). Differentiation between cellular apoptosis and necrosis by the combined use of in situ tailing and nick translation techniques. Lab Invest *71*, 219-25.

González, J., Valls, N., Brito, R., Rodrigo, R. (2014). Essential hypertension and oxidative stress: New insights. World J Cardiol *6*, 353-66.

Gosmanova, E. O., and Le, N. A. (2011). Cardiovascular Complications in CKD Patients: Role of Oxidative Stress. Cardiol Res Pract *2011*, 156326.

Graham, U., Magee, G., Hunter, S., Atkinson, A. (2010). Diabetic nephropathy and chronic kidney disease at a busy diabetes clinic: A study of Outpatient Care and suggestions for improved care pathways at a subspecialty specialist diabetic renal clinic. The Ulster Medical Journal *79*, 57–61.

Grasl-Kraupp, B., Ruttkay-Nedecky, B., Koudelka, H., Bukowska K., Bursch, W., Schulte-Hermann, R. (1995). In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology *21*, 1465-1468.

Grethe, S., Ares, M. P., Andersson, T., Porn-Ares, M. I. (2004). P38 MAPK mediates TNF-induced apoptosis in endothelial cells via phosphorylation and downregulation of Bcl-x(L). Exp. Cell Res *298*, 632-642.

Griendling, K. K., Sorescu, D., Ushio-Fukai, M. (2000). NAD(P)H oxidase: role in cardiovascular biology and disease. Circ Res *86*, 494–501.

Gum, R. J., McLaughlin, M. M, Kumar, S., Wang, Z., Bower, M. J., Lee, J. C. (1998). Acquisition of sensitivity of stress-activated protein kinases to the p38 inhibitor, SB 203580,

by alteration of one or more amino acids within the ATP binding pocket. J. Biol. Chem *273*, 15605-15610.

Gupta, A., Zhuo, J., Zha, J., Reddy, S., Olp, J., Pai, A. (2010). Effect of different intravenous iron preparations on lymphocyte intracellular reactive oxygen species generation and subpopulation survival. BMC Nephrol *17*, 11-16.

Gutteridge, J. M. C., and Halliwell, B. (1994). Antioxidants in nutrition, health and disease. New York: Oxford University Press, 24-39.

Hackenhaar, F. S., Fumagalli, F., Li Volti, G., Sorrenti, V., Russo, I., Staszewsky, L., Masson, S., Latini, R., Ristagno, G. (2014). Relationship between post-cardiac arrest myocardial oxidative stress and myocardial dysfunction in the rat. J Biomed Sci *19*, 21-70.

Haimovitz-Friedman, A., Cordon-Cardo, C., Bayoumy, S., Garzotto, M., McLoughlin, M., Gallily, R., Edwards, C. Kr., Schuchman, E. H., Fuks, Z., Kolesnick, R. (1997). Lipopolysaccharide induces disseminated endothelial apoptosis requiring ceramide generation. J Exp Med *186*, 1831–41.

Halliwell, B. (1993). The role of oxygen radicals in human disease, with particular reference to the vascular system. Haemostasis *23*, 118–26.

Halliwell, B., Clement, M. V., and Long, L. H. (2000). Hydrogen peroxide in the human body. FEBS Lett *486*, 10–13.

Hamza, S. M., and Dyck, J. R. (2014). Systemic and renal oxidative stress in the pathogenesis of hypertension: modulation of long-term control of arterial blood pressure by resveratrol. Front Physiol *5*, 292.

Hansson, G. K. (2005). Inflammation, atherosclerosis, and coronary artery disease.N Engl J Med *352*, 1685-95.

Hasegawa, T., Bragg-Gresham, J. L., Pisoni, R. L., Robinson, B. M., Fukuhara, S., Akiba, T., Saito, A., Kurokawa, K., Akizawa, T. (2011). Changes in anemia management and haemoglobin levels following revision of a bundling policy to incorporate recombinant human erythropoietin. Kidney Int *79*, 340-6.

Hattori, Y., Kawasaki, H., Abe, K., and Kanno, M. (1991). SOD recovers altered endothelium-dependent relaxation in diabetic rat aorta. Am J Physiol *261*, 1086-1094.

Heath, C. W., Strauss, M. B., and Castle, W. B. (1932). QUANTITATIVE ASPECTS OF IRON DEFICIENCY IN HYPOCHROMIC ANAEMIA: (The Parenteral Administration of Iron). J Clin Invest *11*, 1293-312.

Heming, N., Lettéron, P., Driss, F., Millot, S., El Benna, J., Tourret, J., Denamur, E., Montravers, P., Beaumont, C., Lasocki, S. (2012). Efficacy and toxicity of intravenous iron in a mouse model of critical care anaemia. Crit Care Med *40*, 2141-8.

Hemmelgarn, B. R., Clement, F., Manns, B. J., Klarenbach, S., James, M. T., Ravani, P. (2009). Overview of the Alberta Kidney Disease Network. BMC Nephrol *10*, 30.

Hensley, K., Robinson, K. A., Gabbita, S. P., Salsman, S., Floyd, R. A. (2000). Reactive oxygen species, cell signalling, and cell injury. Free Radic Biol Med *28*, 1456-62.

Herrera, J., Nava, M., Romero, F., Rodriquez-Iturbe, B. (2001). Melatonin prevents oxidative stress resulting from iron and erythropoietin administration. Am J Kidney Dis *37*, 750-757.

Higashi, Y., Noma, K., Yoshizumi, M., Kihara, Y. (2009). Endothelial function and oxidative stress in cardiovascular diseases. Circ J *73*, 411-8.

Highsmith, R. F. (1998). Endothelin: molecular biology, physiology, and pathology. Totowa, N.J. HUMAN Press *25*, 91–93.

Hilliwell, B., and Gutterige, J. M. (1999). Free Radicals in biology and medicine. 2nd ed, Oxford University Press, New York.

Himmelfarb, J., Stenvinkel, P., Ikizler, T. A., Hakim, R. M. (2002). The elephant in uremia: oxidant stress as a unifying concept of cardiovascular disease in uremia. Kidney Int *62*, 1524-1538.

Hodges, V. M., Rainey, S., Lappin, T.R., and Maxwell, A. P. (2007). Pathophysiology of anaemia and erythrocytosis. Crit Rev Oncol/Hematol *64*, 139-158.

Holley, A. K., Bakthavatchalu, V., Velez-Roman, J. M., St Clair, D. K. (2011). Manganese Superoxide Dismutase: Guardian of the Powerhouse. Int J Mol Sci *12*, 7114-7162.

Hörl, W. H. (2007). Clinical aspects of iron use in the anemia of kidney disease. J Am Soc Nephrol *18*, 382-93.

Hsieh, H. J., Cheng, C. C., Wu, S. T., Chiu, J. J, Wung, B. S, Wang, D. L. (1998). Increase of reactive oxygen species (ROS) in endothelial cells by shear flow and involvement of ROS in shear-induced c-fos expression. J Cell Physiol *175*, 156-62.

Hsieh, H. J., Liu, C. A., Huang, B., Tseng, A. H., Wang, D. L. (2014). Shear-induced endothelial mechanotransduction: the interplay between reactive oxygen species (ROS) and nitric oxide (NO) and the pathophysiological implications. J Biomed Sci *13*, 21-3.

Hsu, C. Y., Propert, K., Xie, D., Hamm, L., He, J., Miller, E. (2011). Measured GFR Does Not Outperform Estimated GFR in Predicting CKD-related Complications. J Am Soc Nephrol *22*, 1931-7.

Hsu, S. P., Chiang, C. K., Yang, S. Y., Chien, C. T. (2010). N-acetylcysteine for the management of anaemia and oxidative stress in hemodialysis patients. Nephron *116*, 207-216.

Huang, C. Y., Ferrell, J.E, Jr. (1996). Ultrasensitivity in the mitogen-activated protein kinase cascade. Proc Natl Acad Sci U S A *93*, 10078-10083.

Ichii, H., Masuda, Y., Hassanzadeh, T., Saffarian, M., Gollapudi, S., & Vaziri, N. D. (2012). Iron Sucrose Impairs Phagocytic Function and Promotes Apoptosis in Polymorphonuclear Leukocytes. American Journal of Nephrology *36*, 50-57.

Ignarro, L. J., Cirino, G., Casini, A. and Napoli, C. (1999). Nitric oxide as a signaling molecule in the vascular system: an overview. J. Cardiovasc. Pharmacol *34*, 879-886.

Iolascon, A., De Falco, L., and Beaumont, C. (2009). Molecular basis of inherited microcytic anaemia due to defects in iron acquisition or haem synthesis. Haematologica *94*, 395-408.

Irani, K. (2000). Oxidant signaling in vascular cell growth, death, and survival: a review of the roles of reactive oxygen species in smooth muscle and endothelial cell mitogenic and apoptotic signaling.Circ Res *87*, 179-83.

Jacobi, J., Maas, R., Arend, M., Cordasic, N., & Hilgers, K. F. (2014). Effect of Lowering Asymmetric Dimethylarginine (ADMA) on Vascular Pathology in Atherosclerotic ApoE-Deficient Mice with Reduced Renal Mass. International Journal of Molecular Sciences *15*, 5522-5535.

Jahn, M. R., Andreasen, H. B., Fütterer, S., Nawroth, T., Schünemann, V., Kolb, U., Hofmeister, W., Muñoz, M., Bock, K., Meldal, M., Langguth, P. (2011). A comparative study of the physicochemical properties of iron isomaltoside 1000 (Monofer), a new intravenous iron preparation and its clinical implications. Eur J Pharm Biopharm *78*, 480-91.

Janssen, W., Pullamsetti, S. S., Cooke, J., Weissmann, N., Guenther, A., Schermuly, R. T. (2013). The role of dimethylarginine dimethylaminohydrolase (DDAH) in pulmonary fibrosis. J Pathol *229*, 242-9.

Jha, H. C., Divya, A., Prasad, J., Mittal, A. (2010). Plasma circulatory markers in male and female patients with coronary artery disease. Heart Lung *39,* 296–303.

Jia, S.J., Jiang, D.J., Hu, C.P., Zhang, X.H., Deng, H.W., Li, Y.J. (2006). Lysophosphatidylcholine-induced elevation of asymmetric dimethylarginine level by the NADPH oxidase pathway in endothelial cells. Vascular Pharmacology 44, 143–148.

Jin, L., Caldwell, R. B., Li-Masters, T., and Caldwell, R. W. (2007). Homocysteine induces EC dysfunction via inhibition of arginine transport. J PhysiolPharmacol *58*, 191-206.

Jones, D. P., Eklow, L., Thor, H., and Orrenius, S. (1981). Metabolisim of hydrogen peroxide in isolated hepatocytes: Relative concentration of catalase and glutathione peroxidise in decomposition of endogenously generated H2O2. Archives of Biochemistry and Biophysics *210*, 505-516.

Joshi, S., Peck, A. B., Khan, S. R. (2013). NADPH oxidase as a therapeutic target for oxalate induced injury in kidneys. Oxid Med Cell Longev *2013*, 462361.

Joss, N., Patel, R., Paterson, K., Simpson, K., Perry, C., Stirling, C. (2007). Anaemia is common and predicts mortality in diabetic nephropathy. Q J Med *100*, 641-647.

Jun, M., Venkataraman, V., Razavian, M., Cooper, B., Zoungas, S., Ninomiya, T., Webster, A. C., Perkovic, V. (2012). Antioxidants for chronic kidney disease. Cochrane Database Syst Rev *10*, 008176.

Junttila, M. R., Li, S. P., Westermarck, J. (2008). Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. FASEB J *22*, 954-65.

Kaiser, R. A., Bueno, O. F., Lips, D. J., Doevendans, P. A., Jones, F., Kimball, T. F., Molkentin, J. D. (2004). Targeted inhibition of p38 mitogen-activated protein kinase antagonizes cardiac injury and cell death following ischemia-reperfusion in vivo. J. Biol. Chem *279*, 15524-15530.

Kajimoto, H., Kai, H., Aoki, H. (2012). Inhibition of eNOS phosphorylation mediates EC dysfunction in renal failure: new effect of asymmetric dimethylarginine. Kidney Int *81*, 762-768.

Kakhlon, O., and Cabantchik, Z. I. (2002). The labile iron pool: characterization, measurement, and participation in cellular processes. Free Radic. Biol. Med *33*, 1037-1046.

Kalantar-Zadeh, K., Braglia, A., Chow, J., Kwon, O., Kuwae, N., Colman, S., Cockram, D. B., Kopple, J. D. (2005). An anti-inflammatory and antioxidant nutritional supplement for hypoalbuminemic hemodialysis patients: a pilot/feasibility study. J Ren Nutr *15*, 318-331.

Kalyanaraman, B. (2013). Teaching the basics of redox biology to medical and graduate students: Oxidants, antioxidants and disease mechanisms. Redox Biology *1*, 244-257.

Kalyanaraman, B., Darley-Usmar, V., Davies, K. J., Dennery, P. A., Forman, H. J., Grisham, M. B., Mann, G. E., Moore, K., Roberts, L. J., Ischiropoulos, H. (2011). Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations.Free RadicBiol Med *52*, 1-6.

Kamanna, V. S., Ganji, S. H., Shelkovnikov, S., Norris, K., Vaziri, N. D. (2012). Iron Sucrose Promotes Endothelial Injury and Dysfunction and Monocyte Adhesion/Infiltration. Am J Nephrol *35,*114-119.

Kamata, H., and Hirata, H. (1999). Redox regulation of cellular signalling.Cell. Signal *11*, 1–14.

Kang, Y. H., Lee, S. J. (2008). The role of p38 MAPK and JNK in Arsenic trioxide-induced mitochondrial cell death in human cervical cancer cells. J Cell Physiol *217*, 23-33.

Kannel, W. B. (2005). Overview of hemostatic factors involved in atherosclerotic cardiovascular disease. Lipids *40*, 1215-1220.

Kanner, J., German, J. B., Kinsella, J. E. (1987). Intiation of lipid peroxidation in biological system, CRC Crit. Rev. Food Sci. Nut *25*, 317-364.

Kari, J. A., Donald, A. E., Vallance, D. T., Bruckdorfer, K. R., Leone, A., Mullen, M. J., Bunce, T., Dorado, B., Deanfield, J. E., Rees, L. (1997). Physiology and biochemistry of endothelial function in children with chronic renal failure. Kidney Int *52*, 468–472.

Karlsson, M., Kurz, T., Brunk, U. T., Nilsson, S. E., Frennesson, C. I. (2010). What does the commonly used DCF test for oxidative stress really show? Biochem. J *428*, 183–190.

Kartikasari, A. E., Georgiou, N. A., Visseren, F. L., van Kats-Renaud, H., van Asbeck, B. S., Marx, J. J. (2006). Endothelial activation and induction of monocyte adhesion by nontransferrin-bound iron present in human sera.FASEB *20*, 353-5.

Kaushal, S., Amiel, G. E., Guleserian, K. J., Shapira, O. M., Perry, T., Sutherland, F. W., Rabkin, E., Moran, A. M., Schoen, F. J., Atala, A., et al. (2001). Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. Nat Med *7,* 1035-1040.

Kausz, A. T., Obrador, G. T., Periera,B. J. (2000). Anaemia management in patients with chronic renal insufficiency.Am J Kidney Dis *36*, 39–51.

Kayode, O., Osungbade., Adeolu, O., Oladunjoye. (2012). Preventive treatments of iron deficiency anaemia in pregnancy: A review of their effectiveness and implications for health system strengthening *2012*, 7.

Keher, J. P. (1993). Free radicals as mediators of tissue injury and disease, Crit. Rev. Toxic *23*, 21-48.

Keith, D., Nicholls, G., Guillion, C., Brown, J. B., and Smith, D. H. (2004). Longitudinal follow-up and outcomes among a population with chronic kidney disease in a large managed care organization. Arch Intern Med *164*, 659–663.

Kell, D. B. (2009). Iron behaving badly: inappropriate iron chelation as a major contributor to the aetiology of vascular and other progressive inflammatory and degenerative diseases. BMC Med *2*, 2.

Kerr, J. F., Wyllie, A. H., Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer *26*, 239-257.

Kerr, M., Bray, B., Medcalf, J. (2012). Estimating the financial cost of chronic kidney disease to the NHS in England. Nephrology Dialysis Transplantation. *27*, 73–80.

Khaira, A., Mahajan, S., Kumar, A., Saraya, A., Tiwari, S. C., Prakash, S., Gupta, A., Bhowmik, D., Agarwal, S. K. (2011). Endothelial function and oxidative stress in chronic kidney disease of varying severity and the effect of acute hemodialysis. Ren Fail *33*, 411-7.

Kim, J. A., Montagnani, M., Koh, K. K., Quon, M. J. (2006). Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. Circulation *113*, 1888-904.

Kim, S. Y., and Moon, A. (2012). Drug-Induced Nephrotoxicity and Its Biomarkers. Biomolecules & Therapeutics *20*, 268–272.

Kinlay, S., Behrendt, D., Wainstein, M., Beltrame, J., Fang, J. C., Creager, M. A., Selwyn, A. P., and Ganz, P. (2001). Role of endothelin-1 in the active constriction of human atherosclerotic coronary arteries. Circulation *104*, 1114-1118.

Kitamura, M., and Fine, L., G. (1999). The concept of glomerular self-defense. Kidney Int *55*, 1639.

Kobayashi, H., Boelte, K. C., Lin, P. C. (2007). Endothelial cell adhesion molecules and cancer progression. Curr Med Chem *14*, 377-86.

Koike, M., and Nitta, K. (2011). Chronic kidney disease and atherosclerosis. Nihon Rinsho *69*, 144-50.

Kolluru, G. K., Bir, S. C., Kevil, C. G. (2012). Endothelial dysfunction and diabetes: effects on angiogenesis, vascular remodeling, and wound healing. Int J Vasc Med 2012, 918267.

Koshy, S.M., and Geary, D. F. (2008). Anemia in children with chronic kidney disease. Pediatr Nephrol *23*, *209*–219.

Kovacic, P., Pozos, R. S., Somanathan, R., Shangari, N., and O'Brien, P. J. (2005). Mechanism of mitochondrial uncouplers, inhibitors, and toxins: Focus on electron transfer, free radicals, and structure–activity relationships. Curr. Med. Chem *12*, 2601–2623.

Kulnigg, S., Gasche, C. (2006). Systematic review: managing anaemia in Crohn's disease. Aliment PharmacolTher *24*, 1507-1523.

Kulnigg, S., Stoinov S., Simanenkov, V., Dudar, L. V. (2008). A novel intravenous iron formulation for treatment of anemia in inflammatory bowel disease: the ferric carboxymaltose (FERINJECT) randomized controlled trial. Am J Gastroenterol *103*, 1182-92.

Kumar, S., Mcdonnel, P.C., Gum, R.J., Hand, A.T., Lee, J.C., and Young, P.R. (1997). Novel homologues of CSBP/p38 MAP kinase: activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. Biochem. Biophys. Res. Commun *235*, 533–538.

Kumar, V., Cotran, R., and Robbins, S. (2003). In: Basic Pathology. 7th ed. Philadelphia, Pa: Saunders: an imprint of Elsevier Science.

Kumbasar, A., Gursu, M., Kaya, C., Ozturk, S., Ergen, A., Kemik, A. (2012). The effect of different doses and types of intravenous iron on oxidative stress and inflammation in hemodialysis patients. J Nephrol 25, 825-32.

Kunsch, C., and Medford, R. M. (1999). Oxidative stress as a regulator of gene expression in the vasculature. Circ Res *85*, 753-766.

Kuo, K. L., Hung, S. C., Lee, T. S., Tarng, D. C. (2014). Iron Sucrose Accelerates Early Atherogenesis by Increasing Superoxide Production and Upregulating Adhesion Molecules in CKD. J Am Soc Nephrol *25*, 2596-606.

Kuo, K. L., Hung, S. C., Lin, Y. P., Tang, C. F., Lee, T. S., Lin, C. P., Tarng, D. C. (2012). Intravenous Ferric Chloride Hexahydrate Supplementation Induced EC dysfunction and Increased Cardiovascular Risk among Hemodialysis Patients. Plos One 7, 50295.

Kuo, K. L., Hung, S. C., Wei, Y. H, Tarng, D. C. (2008). Intravenous iron exacerbates oxidative DNA damage in peripheral blood lymphocytes in chronic hemodialysis patients. J Am Soc Nephrol *19*, 1817–1826.

Kuzkaya, N., Weissmann, N., Harrison, D. G., Dikalov, S. (2003). Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. J Biol Chem *278*, 22546-54.

Kyrylkova, K., Kyryachenko, S., Leid, M., Kioussi, C. (2012). Detection of apoptosis by TUNEL assay. Methods Mol Biol *887*, 41-7.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 156-160.

Lahera, V., Goicoechea, M., de Vinuesa, S. G., Oubiña, P., Cachofeiro, V., Gómez-Campderá, F., Amann, R., Luño, J. (2006). Oxidative stress in uremia: the role of anemia correction. J Am Soc Nephrol *17*, 174-7.

Lake, G. J., and Ridker, P. M. (2002). Inflammatory bio-markers and cardiovascular risk prediction. J Intern Med *252,* 283-294.

Landmesser, U., Dikalov, S., Price, S. R., McCann, L., Fukai, T., Holland, S. M., Mitch, W. E., and Harrison, D. G. (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J. Clin. Invest *111*, 1201-1209.

Larson, C. S., Leip, M. G., Culleton, E. P., Wilson, B., and Levy, P. W. D. (2004). Predictors of new-onset kidney disease in a community-based population. J Am Med Assoc *291*, 844–850.

Lawrence, I. G., Price, D. E., Howlett, T. A., Harris, K. P., Feehally, J., and Walls, J. (1997). Erythropoietin and sexual dysfunction. Nephrol Dial Transplant *12*, 741-7.

Lea, J. P., and Nicholas, S. B. (2002). Diabetes mellitus and hypertension: key risk factors for kidney disease. J Natl Med Assoc *94*, 7–15.

Lee, D. W., Andersen, J. K., Kaur, D. (2006a). Iron dysregulation and neurodegeneration: the molecular connection. Mol. Intervent *6*, 89–97.

Lee, H. Y., Chae, I. H., Kim, H. S., Park, Y. B., Choi, Y. S., Lee, Y. W., Park, S. J., and Cha, Y. J. (2002). Differential effects of homocysteine on porcine endothelial and vascular smooth muscle cells. J CardiovascPharmacol *39*, 643-651.

Lee, S. H., Meng, X. W., Flatten, K. S., Loegering, D. A., Kaufmann, S. H. (2013). Phosphatidylserine exposure during apoptosis reflects bidirectional trafficking between plasma membrane and cytoplasm. Cell Death Differ *20*, 64-76.

Leehey, D. J., Palubiak, D. J., Chebrolu, S., and Agarwal, R. (2005). Sodium ferric gluconate causes oxidative stress but not acute renal injury in patients with chronic kidney disease: A pilot study. Nephrol Dial Transplant *20*, 135- 140.

Leopold, J. A., and Loscalzo, J. (2009). Oxidative risk for atherothrombotic cardiovascular disease. Free Radic Biol Med *47*, 1673-1706.

Levey, A. S., Atkins, R., and Coresh, J. (2007). Chronic kidney disease as a global public health problem: approaches and initiatives— a position statement from Kidney Disease Improving Global Outcomes. Kidney Int *72*, 247–259.

Levey, A. S., Beto, J. A., Coronado, B. E., (1998). Controlling the epidemic of cardiovascular disease in chronic renal disease: what do we know? What do we need to learn? Where do we go from here? National Kidney Foundation Task Force on Cardiovascular Disease. Am J Kidney Dis *32*, 853–906.

Levey, A. S., Eckardt, K. U., Tsukamoto, Y., Levin, A., Coresh, J., Rossert, J., Zeeuw, D., Hostetter, T. H., Lameire, N., Eknoyan, G. (2005). Definition and classification of chronic kidney disease: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). Kidney Int *67,* 2089-2100.

Levey, A., and Eknoyan, G. (1999). Cardiovascular disease in chronic renal disease. Nephrol Dial Transplant *14*, 828–833.

Levin, A., Thompson, C. R., and Ethier, J. (1999). Left ventricular mass index increase in early renal disease: impact of decline in haemoglobin. Am J Kidney Dis *34*, 125-134.

Li, J. M., and Shah, A. M. (2004). Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. Am. J. Physiol. Regul. Intergr. Comp. Physiol *287*, 2014-1030.

Li, J., and Yuan, J. (2008). Caspases in apoptosis and beyond. Oncogene *27*, 6194-6206.

Li, L., Chen, W., Rezvan, A., Jo, H., Harrison, D. G. (2011). Tetrahydrobiopterin deficiency and nitric oxide synthase uncoupling contribute to atherosclerosis induced by disturbed flow. Arterioscler. Thromb. Vasc. Biol *31*, 1547-1554.

Li, W. M., Liu, H. T., Li, X. Y., Wu, J. Y., Xu, G., Teng, Y. Z., Ding, S. T., Yu, C. (2010).The effect of tetramethylpyrazine on hydrogen peroxide-induced oxidative damage in human umbilical vein endothelial cells. Basic Clin Pharmacol Toxicol *106*, 45-52.

Libby, P. (2002). Inflammation in atherosclerosis. Nature *420*, 868-874.

Libby, P., Ridker, P. M., Maseri, A. (2002). Inflammation and atherosclerosis. Circulation *105*, 1135-43.

Libetta, C., Sepe, V., Esposito, P., Galli, F., Dal Canton, A. (2011). Oxidative stress and inflammation: implications in uremia and hemodialysis. Clinical Biochemistry *44*, 1189–1198.

Lim, J. H., Woo, J. S., Shin, Y.W. (2009). Cilostazol Protects Endothelial Cells Against Lipopolysaccharide-Induced Apoptosis Through ERK1/2- and P38 MAPK-Dependent Pathways. The Korean Journal of Internal Medicine *24*,113–122.

Lim, P. S., Wei, Y. H., Yu, Y. L., Kho, B. (1999). Enhanced oxidative stress in haemodialysis patients receiving intravenous iron therapy.Nephrol Dial Transplant *14*, 2680–2687.

Lin, K. Y., Ito, A., Asagami, T., Tsao, P. S., Adimoolam, S., Kimoto, M. (2002). Impaired nitric oxide synthase pathway in diabetes mellitus: role of asymmetric dimethylarginine and dimethylarginine dimethylaminohydrolase. Circulation *106*, 987-92.

Lin, W., Huang, Y.W., Zhou, X.D., Ma, Y. (2006). In vitro toxicity of silica nanoparticles in human lung cancer cells. Toxicology and Applied Pharmacology *217*, 252–259.

Lipinski, P., and Drapier, J. C. (1997). Interplay between ferritin metabolism, reactive oxygen species and nitric oxide. J Biol Chem *2*, 559-66.

Liu, H., Chen, X., Han, Y., Li, C., Chen, P., Su, S., Zhang, Y., Pan, Z. (2014). Rho kinase inhibition by fasudil suppresses lipopolysaccharide-induced apoptosis of rat pulmonary microvascular endothelial cells via JNK and p38 MAPK pathway. Biomed Pharmacother *68*, 267-75.

Loirand, G., Sauzeau, V., Pacaud, P. (2013). Small G proteins in the cardiovascular system: physiological and pathological aspects. Physiol Rev *93*, 1659-720.

Loreto, C., Almeida, L .E., Trevilatto, P., Leonardi, R. (2011). Apoptosis in displaced temporomandibular joint disc with and without reduction: an immunohistochemical study. J Oral Pathol Med *40*, 103-10.

Loreto, C., La Rocca, G., Anzalone, R., Caltabiano, R. (2014). The role of intrinsic pathway in apoptosis activation and progression in Peyronie's disease. Biomed Res Int *2014*, 616149.

Loscalzo, J., and Welch, G. (1995). Nitric oxide and the cardiovascular system. Prog Cardiovasc Dis *38*, 87-104.

Lu, T. M., Chung, M. Y., Lin, C. C., Hsu, C. P., Lin, S. J. (2011). Asymmetric dimethylarginine and clinical outcomes in chronic kidney disease. Clin J Am Soc Nephrol *6*, 1566-72.

Luczak, M., Formanowicz, D., Pawliczak, E., Wanic-Kossowska, M., Wykretowicz, A., Figlerowicz, M. (2011). Chronic kidney disease-related atherosclerosis - proteomic studies of blood plasma. Proteome Sci *13*, 9-25.

Lum, H., and Roebuck, K. A. (2001). Oxidant stress and endothelial cell dysfunction. Am J Physiol Cell Physiol *280*, 719-41.

Lusis, A. J. (2000). Atherosclerosis. Nature *407*, 233-241.

Lyseng-Williamson, K. A., Keating, G. M. (2009). Ferric carboxymaltose: a review of its use in iron-deficiency anaemia. Drugs *69*, 739-756.

Macdougall, I. C., Bock, A. H., Carrera, F., Eckardt, K. U., Gaillard, C. (2014). FIND-CKD: a randomized trial of intravenous ferric carboxymaltose versus oral iron in patients with chronic kidney disease and iron deficiency anaemia. Nephrol Dial Transplant *29*, 2075-84.

Macdougall, I. C., Eckardt, K. U., Locatelli, F., and Latest, U. S. (2007). KDOQI Anaemia Guidelines update–what are the implications for Europe? Nephrol Dial Transplant *22*, 2738–2742.

Mäkinen, V. P., Civelek, M., Meng, Q., Zhang, B., Zhu, J., Levian, C., Huan, T., Segrè, A. V. (2014). Integrative genomics reveals novel molecular pathways and gene networks for coronary artery disease. PLoS Genet *10*, 1004502.

Malindretos, P., Sarafidis, P. A., Rudenco, I., Raptis, V., Makedou, K., Makedou, A., Grekas, D. M. (2007). Slow intravenous iron administration does not aggravate oxidative stress and inflammatory biomarkers during hemodialysis: a comparative study between iron sucrose and iron dextran. Am J Nephrol *27*, 572-9.

Mallat, Z., and Tedgui, A. (2000). Apoptosis in the vasculature: mechanisms and functional importance. British Journal of Pharmacology *130*, 947–962.

Malyszko, J. (2010). Mechanism of EC dysfunction in chronic kidney disease. Clin Chim Acta *411*, 1412-20.

Markou, T., Dowling, A. A., Kelly, T., Lazou, A. (2009). Regulation of Bcl-2 phosphorylation in response to oxidative stress in cardiac myocytes. Free Radic Res *43*, 809-16.

Marsh, D. J. (1983). Renal Physiology. Raven Press New York 122-136.

Martens, C. R., and Edwards, D. G. (2011). Peripheral Vascular Dysfunction in Chronic Kidney Disease. Cardiology Research and Practice *2011*, 267257.

Martin-Malo, A., Merino, A., Carracedo, J., Alvarez-Lara, M. A., Ojeda, R., Soriano, S., Crespo, R., Ramirez, R., Aljama, P. (2012). Effects of intravenous iron on mononuclear cells during the haemodialysis session. Nephrol Dial Transplant *6*, 2465-71.

Masuda, Y., Ichii H., Vaziri, N. D. (2013). At pharmacologically relevant concentrations intravenous iron preparations cause pancreatic beta cell death. Am J Transl Res *6*, 64-70.

McClellan, W. M., and Flanders, W. D. (2003). Risk factors for progressive chronic kidney disease. J Am Soc Nephrol *14*, 65-70.

McClellan, W. M., Flanders, W. D., Langston, R. D., Jurkovitz, C., Presley, R. (2002). Anaemia and renal insufficiency are independent risk factors for death among patients with

congestive heart failure admitted to community hospitals: a population-based study.J Am SocNephrol *13*, 928-1936.

McClellan, W., Aronoff, S. L., Bolton, W. K. (2004). The prevalence of anaemia in patients with chronic kidney disease. Curr Med Res Opin *20*, 1501-10.

McCord, J. M., Sem. (1998). Iron free radicals, and oxidative injury. Hematology *35*, 5-12.

McFarlane, S. I., Chen, S. C., Whaley-Connell, A. T. (2008). Prevalence and associations of anaemia of CKD: Kidney Early Evaluation Program (KEEP) and National Health Nutrition Examination Survey (NHANES) 1999-2004. Am J Kidney Dis *51*, 46-55.

McGill, H. C. Jr., McMahan, C. A., Tracy, R. E., Oalmann, M. C., Cornhill, J. F., Herderick, E. E., and Strong, J. P. (1998). Relation of a postmortem renal index of hypertension to atherosclerosis and coronary artery size in young men and women. Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group. Arterioscler Thromb Vasc Biol *18*, 1108–1118.

McLaughlin, M. J., and Courtney, A. E. (2013). Early recognition of CKD can delay progression. Practitioner *257*, 13-7.

McMahon, L. P. (2010). “Iron deficiency in pregnancy,” Obstetric Medicine *3*, 17-24.

Mehdi, U., and Toto, R. D. (2009). Anemia, diabetes, and chronic kidney disease. Diabetes Care *32*, 1320-6.

Mello Fiho, A. C., Meneghini, R. (1984). In vivo formation of single-strand breaks in DNA by hydrogen peroxide is mediated by the Haber-Weiss reaction, Biochem. Biophys. Acta 781, 56-63.

Memon, L., Spasojevic-Kalimanovska, V., Bogavac-Stanojevic, N., Kotur-Stevuljevic, J., Simic-Ogrizovic, S. (2013). Assessment of EC dysfunction: the role of symmetrical dimethylarginine and proinflammatory markers in chronic kidney disease and renal transplant recipients. Dis Markers *35*, 173-80.

Messner, B., and Bernhard, D. (2014). Smoking and cardiovascular disease: mechanisms of EC dysfunction and early atherogenesis. Arterioscler Thromb Vasc Biol *34*, 509-15.

Mestas, J., and Ley, K. (2008). Monocyte-endothelial cell interactions in the development of atherosclerosis. Trends Cardiovasc Med *18*, 228-32.

Meyer, M., Pahl, H, L., Baeuerle, P. A. (1994). Regulation of the transcription factors NF-Kappa B and AP-1 by redox changes. Chem Bio Interact *91*, 91-100.

Mezzogiorno, A., Mezzogiorno, V., Esposito, V. (2002). History of the nephron. Am J Nephrol *22*, 213.

Middleton, R. J., Foley, R. N., Hegarty, J., Cheung, C. M., McElduff, P., Gibson, J. M., Kalra, P. A., O'Donoghue, D. J., and New, J. P. (2006). The unrecognized prevalence of chronic kidney disease in diabetes. Nephrol Dial Transplant *21*, 88–92.

Milioti, N., Bermudez-Fajardo, A., Penichet, M. L., Oviedo-Orta, E. (2008). Antigen-Induced Immunomodulation in the Pathogenesis of Atherosclerosis. Clinical and Developmental Immunology *2008*, 723539.

Miller, D. M., Buettner, G. R., Aust, S. D. (1990). Transition metals as catalysts of "autoxidation" reactions. Free Radic. Biol. Med *8*, 95–108.

Moe, S. M., O'Neill, K. D., Fineberg, N., Persohn, S., Ahmed, S., Garrett, P., Meyer, C. A. (2003). Assessment of vascular calcification in ESRD patients using spiral CT. Nephrol Dial Transplant *18*, 1152-8.

Moore, R. A., Gaskell, H., Rose, P., Allan, J. (2011). Meta-analysis of efficacy and safety of intravenous ferric carboxymaltose (Ferinject) from clinical trial reports and published trial data. BMC Blood Disord *24*, 11-4.

Moreno, F., Sanz-Guajardo, D., Lopez-Gomez, J. M., Jofre, R., Valderrabano, F. (2000). Increasing the hematocrit has a beneficial effect on quality of life and is safe in selected hemodialysis patients. Spanish Cooperative Renal Patients Quality of Life Study Group of the Spanish Society of Nephrology. J Am Soc Nephrol *11*, 335–342.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods *65*, 55-63.

Muñoz-Pinedo, C. (2012). Signaling pathways that regulate life and cell death: evolution of apoptosis in the context of self-defense. Adv Exp Med Biol *738*, 124-43.

Najjar, S., Pearlman, D. M., Devinsky, O., Najjar, A., Zagzag, D. (2013). Neurovascular unit dysfunction with blood-brain barrier hyperpermeability contributes to major depressive disorder: a review of clinical and experimental evidence. J Neuroinflammation *1*, 10-142.

Nakano, T., Ninomiya, T., Sumiyoshi, S. (2010). Association of kidneyfunction with coronary atherosclerosis and calcification in autopsy samples from Japanese elders: the Hisayama study. Am J Kidney Dis *55*, 21–30.

Napoli, C., de Nigris, F., Williams-Ignarro, S. (2006). Nitric Oxide and atherosclerosis: An update. Nitric Oxide *15*, 265-279.

Natarajan, V., Scribner, W. M., Morris, A. J., Roy, S., Vepa, S., Yang, J., Wandgaonkar, R., Reddy, S. P. M., Garcia, J. G. N., Parinandi, N. L. (2001). Role of p38 MAP kinase in diperoxovanadate-induced phospholipase D activation in endothelial cells. Am J Physiol Lung Cell Mol Physiol *281*, 435-449.

National Institute for Health and Clinical Excellence (NICE). (2012). National clinical guideline for early identification and management in adults.

National Institute for Health and Clinical Excellence (NICE). (2014). Lipid modification: cardiovascular risk assessment and the modification of blood lipids for the primary and secondary prevention of cardiovascular disease.

National Institute for Health and Clinical Excellence (NICE): Chronic Kidney Disease. (2014). National clinical guideline for early identification and management in adults in primary and secondary care.

National Kidney Foundation. (2002). K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis *39*, 1-266.

Nelson, S. K., and McCord, J. M. (1998). Iron, oxygen radicals, and disease. Adv Mol Cell Biol *25*, 157-83.

Nencioni, L., De Chiara, G., Sgarbanti, R., Amatore, D., Aquilano, K., Marcocci, M. E., Palamara, A. T. (2009). Bcl-2 Expression and p38MAPK Activity in Cells Infected with Influenza A Virus: IMPACT ON VIRALLY INDUCED APOPTOSIS AND VIRAL REPLICATION. The Journal of Biological Chemistry *28,* 16004–16015.

New, J. P., Aung, T., Baker, P. G., Yongsheng, G., Pylypczuk, R., Houghton, J., Rudenski, A., New, R. P. (2008). The high prevalence of unrecognized anaemia in patients with diabetes and chronic kidney disease: a population-based study. Diabet Med *25*, 564– 569.

Nissenson, A. R. (1991). National cooporative rHuEPO study in patients with chronic renal failure: A phase IV Multicenter study. Am J Kidney Dis *18*, 24-33.

Norbury, C. J., and Hickson, I. D. (2001). Cellular responses to DNA damage.Annu Rev PharmacolToxicol *41*, 367-401.

Nordberg, J., and Arner, E. S. (2001). Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic. Biol. Med *31*, 1287-1312.

Nordfjeld, K., Andreasen H., Thomsen, L. L. (2012). Pharmacokinetics of iron isomaltoside 1000 in patients with inflammatory bowel disease. Drug Des Devel Ther *6*, 43-51.

O'Hare, A. M., Glidden, D. V., Fox, C. S., Hsu, C. Y. (2004). High prevalence of peripheral arterial disease in persons with renal insufficiency: results from the National Health and Nutrition Examination Survey 1999–2000. Circulation *109*, 320–323.

Oberg, B. P., McMenamin, E., Lucas, F. L., McMonagle, E., Morrow, J., Ikizler, T. A., Himmelfarb, J. (2004). Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease.Kidney Int *65,* 1009-1016.

Ogino, K., Wang, D. H. (2007). Biomarkers of oxidative/nitrosative stress: an approach to disease prevention. Acta Med Okyama *61*, 181- 9.

Oliveira, M. B., Romao, J. E. J., Zatz, R. (2005). End-stage renal diasease in Brazil: epidemiology, prevention, and treatment. Kidney Int Suppl *97*, 82-86.

Omae, T., Nagaoka, T., Tanano, I., Yoshida, A. (2013). Homocysteine inhibition of endothelium-dependent nitric oxide-mediated dilation of porcine retinal arterioles via enhanced superoxide production. Invest Ophthalmol Vis Sci *54*, 2288-95.

Ono, K., and Han, J. (2000). The p38 signal transduction pathway: activation and function. Cell. Signal *12*, 1-13.

Orita, Y., Nishizaki, K., Sasaki, J., Kanda, S., Kimura, N., Nomiya, S., Yuen, K., Masuda, Y. (1999). Does TUNEL staining during peri- and post-natal development of the mouse inner ear indicate apoptosis? ActaOtolaryngolSuppl *540*, 22-26.

Osone, S., Hosoi, H., Kuwahara, Y., Matsumoto, Y., Iehara, T., Sugimoto, T. (2004).Fenretinide induces sustained-activation of JNK/p38 MAPK and apoptosis in a reactive oxygen species-dependent manner in neuroblastoma cells. Int J Cancer *112*, 219–224.

Outinen, P. A., Sood, S. K., Pfeifer, S. I., Pamidi, S., Podor, T. J., Li, J., Weitz, J. I., and Austin, R. C. (1999). Homocysteine-induced endoplasmic reticulum stress and growth arrest leads to specific changes in gene expression in human vascular endothelial cells. Blood *94*, 959-967.

Pacurari, M., Xing, D., Hilgers, R. H. P., Guo, Y. Y., Yang, Z., & Hage, F. G. (2013). Endothelial cell transfusion ameliorates EC dysfunction in 5/6 nephrectomized rats. American Journal of Physiology - Heart and Circulatory Physiology *305*, 1256-1264.

Pai, A. B., Boyd, A. V., McQuade, C. R., Harford, A., Norenberg, J. P., and Zager, P. G. (2007). Comparison of oxidative stress markers after intravenous administration of iron dextran, sodium ferric gluconate, and iron sucrose in patients undergoing hemodialysis *27*, 343-50.

Pai, A. B., Conner, T., McQuade, C. R., Olp, J., Hicks, P. (2011).Non-transferrin bound iron, cytokine activation and intracellular reactive oxygen species generation in hemodialysis patients receiving intravenous iron dextran or iron sucrose. Biometals *24*, 603-613.

Papatheodorou, L., and Weiss, N. (2007). Vascular oxidant stress and inflammation in hyperhomocysteinemia. Antioxid Redox Signal *9*, 1941-1958.

Papayianni, A., Alexopoulos, E., Giamalis, P., Gionanlis, L., Belechri, A. M., Koukoudis, P., Memmos, D. (2002). Circulating levels of ICAM-1, VCAM-1, and MCP-1 are increased in haemodialysis patients: association with inflammation, dyslipidaemia, and vascular events. Nephrol Dial Transplant *17*, 435–441.

Paravicini, T. M., and Touyz, R. M. (2008). NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities. Diabetes Care *31*, 170-180.

Park, G. B., Choi, Y., Kim, Y. S., Lee, H. K., Kim, D., Hur, D. Y. (2014). ROS-mediated JNK/p38-MAPK activation regulates Bax translocation in Sorafenib-induced apoptosis of EBV-transformed B cells. Int J Oncol *44*, 977-85.

Park, H. S., Hwang, H. J., Kim, G. Y., Cha, H. J., Kim, W. J., Kim, N. D., Yoo, Y. H., Choi, Y. H. (2013). Induction of apoptosis by fucoidan in human leukemia U937 cells through activation of p38 MAPK and modulation of Bcl-2 family. Mar Drugs *11*, 2347-64.

Park, M., Hsu, C. Y., Li, Y., Mishra, R. K., Keane, M., Rosas, S. E., Dries, D., Xie, D. (2012). Associations between kidney function and subclinical cardiac abnormalities in CKD. J Am Soc Nephrol *23*, 1725-34.

Parker, J. (1998). Contemporary Nephrology Nursing. New Jersey: American Nephrology Nurses' Association.

Passauer, J., Bussemaker, E., Range, U., Plug, M., Gross, P. (2000). Evidence in vivo showing increase of baseline nitric oxide generation and impairment of endothelium-dependent vasodilation in normotensive patients on chronic hemodialysis. J Am SocNephrol *11*, 1726–1734.

Patterson, C., Ruef, J., Madamanchi, N. R., Barry-Lane, P., Hu, Z., Horaist, C., Ballinger, C. A., Brasier, A. R., Bode, C., Runge, M. S. (1999). Stimulation of a vascular smooth muscle cell NAD(P)H oxidase by thrombin. Evidence that p47(phox) may participate in forming this oxidase in vitro and in vivo. J Biol Chem *274*, 19814-19822.

Pearson, G., Robinson, F., Beers Gibson,T., Xu, B. E., Karandikar, M., and Cobb, M. H. (2001). Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. Endocr. Rev *22*, 153-183.

Pearson, T. A. (1999). Cardiovascular disease in developing countries: myth, realties, and opportunities. Cardiovascular Drugs and Tearapy *13*, 95-104.

Pecoits-Filho, R., Heimburger, O., Barany, P., Suliman, M., Fehrman-Ekholm, I., Lindholm, B., Stenvinkel, P. (2003). Associations between circulating inflammatory markers and residual renal function in CRF patients.Am J Kidney Dis *41*, 1212 –1218.

Pellegrini, L and Scorrano, L. (2007). A cut short to death: Parl and Opa1 in the regulation of mitochondrial morphology and apoptosis. Cell Death Differ *14*, 1275-1284.

Perk, J., De Backer, G., Gohlke, H., Graham, I., Reiner, Z. (2012). European Guidelines on cardiovascular disease prevention in clinical practice (version 2012): The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of nine societies and by invited experts). Atherosclerosis *223*, 1-68.

Perticone, F., Maio, R., Perticone, M., Sciacqua, A., Shehaj, E., Naccarato, P., Sesti, G. (2010). “EC dysfunction and subsequent decline in glomerular filtration rate in hypertensive patients,” Circulation *122*, 379–384.

Pescador, R., Capuzzi, L., Mantovani, M., Fulgenzi, A., Ferrero, M. E. (2013). Defibrotide: properties and clinical use of an old/new drug. Vascul Pharmacol *1-2,* 1-10.

Peter, V, Usatyuk, Suryanarayana Vepa, Tonya Watkins, Donghong He, Narasimham L. Parinandi, Viswanathan Natarajan. (2003). Redox Regulation of Reactive Oxygen Species-Induced p38 MAP Kinase Activation and Barrier Dysfunction in Lung Microvascular Endothelial Cells Antioxidants & Redox Signaling *5*, 723-730.

Phinikaridou, A., Andia, M. E., Protti, A., Indermuehle, A., Shah, A., Smith, A., Warley, A., Botnar, R. M. (2012). Noninvasive magnetic resonance imaging evaluation of endothelial permeability in murine atherosclerosis using an albumin-binding contrast agent. Circulation *126*, 707-19.

Pirillo, A., Norata, G. D., Catapano, A. L. (2013). LOX-1, OxLDL, and atherosclerosis. Mediators Inflamm *2013*, 152786.

Plotnikov, A., Zehorai, E., Procaccia, S., Seger, R. (2011). The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. Biochim Biophys Acta *1813*, 1619-33.

Pober, J. S., and Sessa, W. C. (2007). Evolving functions of endothelial cells in inflammation.Nat Rev Immunol *7*, 803-15.

Porras, A., Zuluaga, S., Black, E., Valladares, A., Alvarez, A. M., Ambrosino, C., Benito, M., Nebreda, A. R. (2004). P38 alpha mitogen-activated protein kinase sensitizes cells to apoptosis induced by different stimuli. MolBiol Cell *15*, 922-33.

Praschberger, M., Cornelius, C., Schitegg, M., Goldenberg, H., Scheiber-Mojdehkar, B., Sturm, B. (2013). Bioavailability and stability of intravenous iron sucrose originator versus generic iron sucrose AZAD. Pharm Dev Technol *20*, 176-82.

Prats, M., Font, R., García-Ruiz, C., Cabré, C., Muñoz-Cortés, M., Nogués, M. R., Jariod, M., Romeu, M., Martínez-Vea, A. (2013). Acute and sub-acute effect of ferric carboxymaltose on inflammation and adhesion molecules in patients with predialysis chronic renal failure.Nefrologia *33*, 355-61.

Price, D. T., and Loscalzo, J. (1999). Cellular adhesion molecules and atherogenesis.Am J Med *107*, 85-97.

Puddu, P., Puddu, G. M., Galletti, L., Cravero, E., Muscari, A. (2005). Mitochondrial dysfunction as an initiating event in atherogenesis: aplausible hypothesis. Cardiology *103*, 137-141.

Qian, S. Y and Buettner, G. R. (1999). Iron and dioxygen chemistry is an important route to initiation of biological and free radical oxidations: an electron paramagnetic resonance spin trapping study. Free Radic. Biol. Med *26*, 1447–1456.

Rahman, K. (2007). Studies on free radicals, antioxidants, and co-factors. Clinical Interventions in Aging *2*, 219-236.

Rahman, M., Pressel, S., Davis, B. R., Nwachuku, C., Wright, J. T. Jr., Whelton, P. K. (2006). Cardiovascular outcomes in high-risk hypertensive patients stratified by baseline glomerular filtration rate. Ann Intern Med *144*, 172-80.

Raitakari, O. T., Adams, M. R., McCredie, R. J., Griffiths, K. A., Stocker, R., Celermajer, D. S. (2000). Oral vitamin C and endothelial function in smokers: short-term improvement, but no sustained beneficial effect. J Am Coll Cardiol *35*, 1616-21.

Rayner, B.S., Duong, T.T., Myers, S.J., Witting, P.K., (2006). Protective effect of a synthetic anti-oxidant on neuronal cell apoptosis resulting from experimental hypoxia reoxygenation injury. J. Neurochem *97*, 211–221.

Rehman, T., Fought, J., & Solomon, R. (2008). N-Acetylcysteine Effect on Serum Creatinine and Cystatin C Levels in CKD Patients. Clinical Journal of the American Society of Nephrology : CJASN *3*, 1610–1614.

Reis, K. A., Guz, G., Ozdemir, H., Erten, Y., Atalay, V., Bicik, Z., Ozkurt, Z. N., Bali, M., Sindel, S. (2005). Intravenous iron therapy as a possible risk factor for atherosclerosis in end-stage renal disease.Int Heart J *46*, 255-64.

Rezzani, R., Favero, G., Stacchiotti, A., Rodella, L. F. (2013). Endothelial and vascular smooth muscle cell dysfunction mediated by cyclophylin A and the atheroprotective effects of melatonin. Life Sci *92*, 875-82.

Ribeiro, G., Roehrs, M., Bairros, A., Moro, A., Charão, M., Araújo, F., Valentini, J., Arbo, M., Brucker, N., Moresco, R., Leal, M., Morsch, V., Garcia, S. C. (2011). N-acetylcysteine on oxidative damage in diabetic rats. Drug Chem. Toxicol *34*, 467–74.

Riccardi, C., and Nicoletti, I. (2006). Analysis of apoptosis by propidium iodide staining and flow cytometry. Nat Protoc 1, 1458-61.

Rocco, M. V., Yan, G., Gassman, J., Lewis, J. B., Ornt, D., Weiss, B., Levey, A. S. (2002). Comparison of causes of death using HEMO Study and HCFA end-stage renal disease death notification classification systems. The National Institutes of Health-funded hemodialysis. Health Care Financing Administration. Am J Kidney Dis *39*, 146–153.

Rock, K. L., and Kono, H. (2008). The inflammatory response to cell death. Annu Rev Pathol *3*, 99-126.

Roebuck, K. A., and Finnegan, A. (1999). Regulation of intercellular adhesion molecule-1 (CD54) gene expression. J Leukoc Biol *66*, 876-88.

Romanoski, C. E., Lee, S., Kim, M. J., Ingram-Drake, L., Plaisier, C. L. (2010). Systems genetics analysis of gene-by-environment interactions in human cells. Am J Hum Genet *86*, 399–410.

Rooyakkers, T. M., Stroes, E. S., Kooistra, M. P., van Faassen, E. E., Hider, R. C., Rabelink, T. J., Marx, J. J. (2002). Ferric saccharate induces oxygen radical stress and EC dysfunction in vivo *32*, 9-16.

Rose, B. A., Force, T., Wang, Y. (2010). Mitogen-activated protein kinase signaling in the heart: angels versus demons in a heart-breaking tale. Physiol Rev *90*, 1507-46.

Rosen, G. M., and Freeman, B. A. (1984). Detection of superoxide generated by endothelial cells. Proc Natl Acad Sci USA *81*, 7269–7273.

Ross, R. (1995). Cell biology of atherosclerosis.Annu Rev Physiol *57*, 791-804.

Roux, P. P., and Blenis, J. (2004). ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev *68*, 320-344.

Rowinsky, E. K. (2005). Targeted induction of apoptosis in cancer management: the emerging role of tumor necrosis factor-related apoptosis-inducing ligand receptor activating agents. J Clin Oncol *23*, 9394-407.

Royall, J. A., and Ischiropoulos, H. (1993). Evaluation of 2',7'-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H2O2 in cultured endothelial cells. Arch Biochem Biophys *302*, 348-355.

Rozen-Zvi, B., Gafter-Gvili, A., Paul, M., Leibovici, L., Shpilberg, O., Gafter, U. (2008). Intravenous versus oral iron supplementation for the treatment of anaemia in CKD: systematic review and meta-analysis. Am J Kidney Dis *52*, 897-906.

Rudijanto, A. (2007). The role of vascular smooth muscle cells on the pathogenesis of atherosclerosis. Acta Med Indones *39*, 86-93.

Salas, A., Shimaoka, M., Chen, S., Carman, C. V., Springer, T. (2002). Transition from rolling to firm adhesion is regulated by the conformation of the I domain of the integrin lymphocyte function-associated antigen-1. J Biol Chem 277, 50255-62.

Samouilidou, E. C., Grapsa, E. J., Kakavas, I., Lagouranis, A., Agrogiannis, B. (2003). Oxidative stress markers and C-reactive protein in end-stage renal failure patients on dialysis. nt Urol Nephrol *35*, 393-7.

Sarkanen, J. R., Mannerström, M., Vuorenpää, H., Uotila, J., Ylikomi, T., Heinonen, T. (2010). Intra-Laboratory Pre-Validation of a Human Cell Based in vitro Angiogenesis Assay for Testing Angiogenesis Modulators. Frontiers in Pharmacology *1*, 147.

Sato, A., Okada, M., Shibuya, K., Watanabe, E., Seino, S., Narita, Y., Shibui, S., Kayama, T., Kitanaka, C. (2014). Pivotal role for ROS activation of p38 MAPK in the control of

differentiation and tumor-initiating capacity of glioma-initiating cells. Stem Cell Res *12*, 119-31.

Savoia, C., Sada, L., Zezza, L., Pucci, L., Lauri, F. M. (2011). Vascular inflammation and endothelial dysfunction in experimental hypertension. Int J Hypertens 2011, 281240.

Scarborough, P. et al (2010). Coronary Heart Disease Statistics . 2010 edition. British Heart Foundation.

Schaller, G., Scheiber-Mojdehkar, B., Wolzt, M., Puttinger, H., Mittermayer, F., Hörl, W. H., Födinger, M., Sunder-Plassmann, G., Vychytil, A. (2005). Intravenous iron increases labile serum iron but does not impair forearm blood flow reactivity in dialysis patients. Kidney Int *68*, 2814-22.

Schechter, A. N., and Gladwin, M. T. (2003). Haemoglobin and the paracrine and endocrine functions of nitric oxide. N Engl J Med *348*, 1483-1485.

Schiffrin, E. L., Lipman, M. L., Mann, JF. E. (2007). Chronic kidney disease.Effects on the cardiovascular system. Circulation *116*, 85-97.

Schlossmann, J., Feil, R., and Hofmann, F. (2003). Signaling through NO and cGMP-dependent protein kinases. Ann Med *35*, 21-27.

Schmidt, H., Schmidt, W., Müller, T.,Böhrer, H., Gebhard, M. M., Martin, E. (1997). N-acetyl-cysteine attenuates endotoxin-induced leukocyte-endothelial cell adhesionand macromolecular leakage in vivo. Crit Care Med *25*, 858-63.

Schmidt, R. J., Yokota, S., Tracy, T. S., Sorkin, M. I., Baylis, C. (1999). "Nitric oxide production is low in end-stage renal disease patients on peritoneal dialysis," American Journal of Physiology *276*, 794-797.

Schmieder, R. E., Mann, J. F, Schumacher, H. (2011). Changes in albuminuria predict mortality and morbidity in patients with vascular disease. J Am Soc Nephrol *22*, 1353-64.

Schnabel, R., and Blankenberg, S. (2007). Oxidative stress in cardiovascular disease: successful translation from bench to bedside?. Circulation. *116*, 1338-40.

Scholze, A., Rinder, C., Beige, J., Riezler, R., Zidek, W., Tepel, M. (2004). Acetylcysteine reduces plasma homocysteine concentration and improves pulse pressure and endothelial function in patients with end-stage renal failure. Circulation *109*, 369-374.

Schutte, B., Nuydens, R., Geerts, H., Ramaekers, F. (1998). Annexin V binding assay as a tool to measure apoptosis in differentiated neuronal cells. J Neurosci Methods *86*, 63-9.

Schutters, K., and Reutelingsperger, C. (2010). Phosphatidylserine targeting for diagnosis and treatment of human diseases. Apoptosis *15*, 1072-1082.

Sebolt-Leopold, J. S., and Herrera, R. (2004). Targeting the mitogen-activated protein kinase cascade to treat cancer. Nat Rev Cancer *4*, 937-947.

Shah, S. V., and Alam, M. G. (2003).Role of iron in atherosclerosis. Am J Kidney Dis *41*, 80-3.

Shah, S.V. (2001). Role of iron in progressive renal disease.Am J Kidney Dis *37*, 30-33.

Shimokawa, H. (1999). Primary EC dysfunction: atherosclerosis. J. Mol. Cell. Cardiol *31*, 23-37.

Shishir Shetty., Christopher, J., Weston., David, H., Adams., Patricia, F., Lalor. (2014). A Flow Adhesion Assay to Study Leucocyte Recruitment to Human Hepatic Sinusoidal Endothelium under Conditions of Shear Stress. J Vis Exp *85*, 51330.

Shou, Y., Li, N., Li, L., Borowitz, J. L., Isom, G. E. (2002). NF-B-mediated upregulation of Bcl-XsandBax contributes to cyotchromecrelease in cyanide-induced apoptosis.J. Neurochem *81*, 842-852.

Showkat, A., Bastnagel, W. R., Hudson, J. Q. (2014). Effect of α -Lipoic Acid on Oxidative Stress in End-Stage Renal Disease Patients Receiving Intravenous Iron.ISRN Nephrol *63*, 45-15.

Sibal, L., Agarwal, S. C., Home, P. D., and Boger, R. H. (2010). The Role of Asymmetric Dimethylarginine (ADMA) in EC dysfunction and Cardiovascular Disease. *Current Cardiology Reviews 6*, 82-90.

Sies, H. (1986). Biochemistry of oxidative stress, Angew. Chem. Int. Ed Engl *25*, 1058-1071.

Sies, H. (1997). Oxidative stress: oxidants and antioxidants. Exp Physiol *82*, 291-295.

Sigaud, S., Evelson, P., Gonzalez-Flecha, B. (2005). H_2O_2-induced proliferation of primary alveolar epithelial cells is mediated by MAP kinases. Antioxid Redox Signal *7*, 6-13.

Silverberg, D. S., Blum, M., Agbaria, Z. (1999). Intravenous iron for the treatment of predialysis anaemia. Kidney Int Suppl *69*, 79–85.

Silverstein, D. M. (2009). Inflammation in chronic kidney disease: role in the progression of renal and cardiovascular disease. Pediatr Nephrol *24,* 1445-52.

Smith, S. C. Jr., Collins, A., Ferrari, R. (2012). Our time: a call to save preventable death from cardiovascular disease (heart disease and stroke). J Am Coll Cardiol *60,* 2343-8.

Snell, R. (1992). Clinical Anatomy. Boston: Little, Brown and Company. 4th edn.

Son, Y., Cheong, Y. K., Kim, N. H., Chung, H. T., Kang, D. G., Pae, H. O. (2011). Mitogen-Activated Protein Kinases and Reactive Oxygen Species: How Can ROS Activate MAPK Pathways? Journal of Signal Transduction *2011*, 792639.

Sorensen, K. E., Celermajer, D. S., Georgakopoulos, D., Hatcher, G., Betteridge, D. J., Deanfield, J. E. (1994). Impairment of endothelium-dependent dilation is an early event in

children with familial hypercholesterolemia and is related to the lipoprotein (a) level. J Clin Invest *93*, 50–5.

Soria, B., and Gauthier, B. R. (2013). Dual Trade of Bcl-2 and Bcl-xL in islet physiology: balancing life and death with metabolism secretion coupling. Diabetes *62*, 18-21.

Spickett, C. M., Reis, A., Pitt, A. R. (2013). Use of narrow mass-window, high-resolution extracted product ion chromatograms for the sensitive and selective identification of protein modifications. Anal Chem *85*, 4621-7.

Spinowitz, B. S., Kausz, A. T., Baptista, J., Noble, S. D, Sothinathan, R., Bernardo, M. V., Brenner, L., Pereira, B. J. (2008). Ferumoxytol for treating iron deficiency anemia in CKD. J Am Soc Nephrol *19*, 1599-605.

Springer, T. A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell *76*, 301-314.

Stancanelli, B., Malatino, L. S., Cataliotti, A., Bellanuova, I., Mallamaci, F., Tripepi, G., Benedetto, F. A., Leonardis, D., Fatuzzo, P., Rapisarda, F., Zoccali, C. (2010). Soluble e-selectin is an inverse and independent predictor of left ventricular wall thickness in end-stage renal disease patients. Nephron Clin Pract *1141*, 74-80.

Stauffer, M.E., Fan, T. (2014). Prevalence of anemia in chronic kidney disease in the United States. PLoS One 9, e84943.

Steffes, M. W., Schmidt, D., and McCrery, R. (2001). Glomerular cell number in normal subjects and in type 1 diabetic patients. Kidney Int *59*, 2104.

Stenvinkel, P., Carrero, J. J., Axelsson, J., Lindholm, B., Heimbürger, O., Massy, Z. (2008). Emerging biomarkers for evaluating cardiovascular risk in the chronic kidney disease patient: how do new pieces fit into the uraemic puzzle? Clin J Am Soc Nephrol *3*, 505-21.

Stenvinkel, P., Wanner, C., Metzger, T., Heimbürger, O., Mallamaci, F., Tripepi, G., Malatino, L., Zoccali, C. (2002). Inflammation and outcome in endstage renal failure: Does female gender constitute a survival advantage? Kidney Int *62*, 1791-1798.

Stout, R. W. (1993). Diabetes and atherosclerosis. Biomed Pharmacother *47*, 1-2.

Strasser, A., O'Connor, L., Dixit, V. M. (2000).Apoptosis signaling.Annu Rev Biochem *69*, 217-245.

Sturm, B., Laggner, H., Ternes, N., Goldenberg, H., Scheiber-Mojdehkar, B. (2005). Intravenous iron preparations and ascorbic acid: effects on chelatable and bioavailable iron. Kidney Int *67*, 1161-70.

Sturm, B., Steinkellner, H., Ternes, N., Goldenberg, H., Scheiber-Mojdehkar, B. (2010). In vitro study on the effects of iron sucrose, ferric gluconate and iron dextran on redox-active iron and oxidative stress. Arzneimittelforschung *60*, 459-65.

Suen, D. F., Norris, K. L., Youle, R. J. (. 2008). Mitochondrial dynamics and apoptosis. Genes Dev *22*, 1577-90.

Suliman, M. E., Qureshi, A. R., Heimburger, O., Lindholm, B., Stenvinkel, P. (2006). Soluble adhesion molecules in end-stage renal disease: a predictor of outcome. Nephrol Dial Transplant *21*, 1603-1610.

Sultan, S.A., Liu, W., Peng, Y., Roberts, W., Whitelaw, D., Graham, A. M. (2015) The Role of Maternal Gestational Diabetes in Inducing Fetal Endothelial Dysfunction. J Cell Physiol *230*, 2695-705.

Sun, J.Y., Zhu, M.Z., Wang, S.W., Miao, S., Xie, Y.H., Wang, J. B., (2007). Inhibition of the growth of human gastric carcinoma in vivo and in vitro by swainsonine.Phytomedicine *14*, 353-359.

Sun, Y., and Oberley, L. W. (1996). Redox regulation of transcriptional activators. Free Radical Biol. Med *21*, 335-348.

Suzuki, H., Katayama, N., Ikuta, Y., Mukai, K., Fujieda, A. (2004). Activities of granulocyte-macrophage colony-stimulating factor and interleukin-3 on monocytes. Am J Hematol *75*, 179-89.

Svensson, C., Part, K., Künnis-Beres, K., Kaldmäe, M., Fernaeus, S. Z., Land, T. (2011). Pro-survival effects of JNK and p38 MAPK pathways in LPS-induced activation of BV-2 cells. Biochem Biophys Res Commun *406*, 488-92.

Swarnalatha, G., Ram, R., Neela, P., Naidu, M. U., Dakshina Murty, K. V. (2010). Oxidative stress in hemodialysis patients receiving intravenous iron therapy and the role of N-acetylcysteine in preventing oxidative stress. Saudi J Kidney Dis Transpl *21*, 852-8.

Tagboto, S., Cropper, L., Turner, J., Pugh-Clarke, K. (2009). The efficacy of a single dose of intravenous ferric carboxymaltose (Ferinject®) on anaemia in a predialysis population of chronic kidney disease patients. J Ren Care *35*, 18-23.

Takacs, P., Kauma, S. W., Sholley, M. M., Walsh, S. W., Dinsmoor, M. J., Green, K. (2001). Increased circulating lipid peroxides in severe preeclampsia activate NF-kappaB and upregulate ICAM-1 in vascular endothelial cells. FASEB J *15,* 279-81.

Takanashi, T., Ogura, Y., Taguchi, H., Hashizoe, M., Honda, Y. (1997). Fluorophotometric quantitation of oxidative stress in the retina in vivo. Invest Ophthalmol Vis Sci *38*, 2721-8.

Tampo, Y., Kotamraju, S., Chitambar, C. R., Kalivendi, S. V., Keszler, A., Joseph, J., Kalyanaraman, B. (2003). Oxidative stress-induced iron signaling is responsible for peroxide-dependent oxidation of dichlorodihydrofluorescein in endothelial cells: role of transferrin receptor-dependent iron uptake in apoptosis. Circ Res *92*, 56-63.

Tan, C. P., Lu, Y. Y., Ji, L. N., Mao, Z. W. (2014). Metallomics insights into the programmed cell death induced by metal-based anticancer compounds. Metallomics *6*, 978-95.

Tan, P. H., Chan, C., Xue, S. A., Dong, R., Ananthesayanan, B., Manunta, M., Kerouedan, C., Cheshire, N. J., Wolfe, J. H., Haskard, D. O., Taylor, K. M., George, A. J. (2004). Phenotypic and functional differences between human saphenous vein (HSVEC) and umbilical vein (HUVEC) endothelial cells.Atherosclerosis *173*, 171-83.

Tarpey, M. M., and Fridovich, I. (2001). Methods of detection of vascular reactive species: nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite. Circ Res *89*, 224-236.

Tepel, M. (2007). N-Acetylcysteine in the prevention of ototoxicity. Kidney Int *72*, 231–2.

Tepel, M., van der Giet, M., Statz, M., Jankowski, J., Zidek, W. (2003). The antioxidant acetylcysteine reduces cardiovascular events in patients with end-stage renal failure: a randomized, controlled trial. Circulation *107*, 992-995.

Thomas, M. C. (2007). Anaemia in diabetes: marker or mediator of microvascular disease? Nat Clin Pract Nephrol *3*, 20-30.

Thomas, R., Kanso, A., Sedor, J. R. (2008). Chronic kidney disease and its complications. Prim Care *35*, 329-44.

Tian, X. Y., Yung, L. H., Wong, W. T., Liu, J., Leung, F. P., Liu, L., Chen, Y. (2012). Bone morphogenic protein-4 induces endothelial cell apoptosis through oxidative stress-dependent p38MAPK and JNK pathway. J Mol Cell Cardiol *52*, 237-44.

Toblli, J. E., Cao, G., Oliveri, L., Angerosa, M. (2011). Assessment of the extent of oxidative stress induced by intravenous ferumoxytol, ferric carboxymaltose, iron sucrose and iron dextran in a nonclinical model. Arzneimittelforschung *61*, 399-410.

Tonelli, M., Wiebe, N., Culleton, B., House, A., Rabbat, C., Fok, M., McAlister, F., and Garg, A. X. (2006). Chronic kidney disease and mortality risk: a systematic review. J Am Soc Nephrol *17*, 2034 –2047.

Torcia, M., De Chiara, G., Nencioni, L., Ammendola, S., Labardi, D., Lucibello, M., Rosini, P., Marlier, L. N., Bonini, P., DelloSbarba, P., Palamara, A. T., Zambrano, N., Russo, T., Garaci, E., Cozzolino, F. (2001). Nerve growth factor inhibits apoptosis in memory B lymphocytes via inactivation of p38 MAPK, prevention of Bcl-2 phosphorylation and cytochrome c release. J BiolChem *276*, 39027-39036.

Torcia, M., De Chiara, G., Nencioni, L., Ammendola, S., Labardi, D. (2001). Nerve growth factor inhibits apoptosis in memory B lymphocytes via inactivation of p38 MAPK, prevention of Bcl-2 phosphorylation, and cytochrome c release. Biol Chem *276,* 39027-36.

Totoson, P., Maguin-Gaté, K., Prati, C., Wendling, D., Demougeot, C. (2014). Mechanisms of EC dysfunction in rheumatoid arthritis: lessons from animal studies. Arthritis Res Ther *16*, 202.

Touyz, R. M., and Schiffrin, E. L. (2004). Reactive oxygen species in vascular biology: implication in hypertention. Histochem Cell Biol *122*, 339.

Trachootham, D., Lu, W., Ogasawara, M. A., Nilsa, R. D., Huang, P. (2008). Redox regulation of cell survival.Antioxid. Redox Signal *10*, 1343-1374.

Trespalacios, F. C., Taylor, A. J., Agodoa, L. Y., Bakris, G. L., Abbott, K. C. (2003). Heart failure as a cause for hospitalization in chronic dialysis patients. Am J Kidney Dis *41*, 1267-77.

Tricot, O., Mallat, Z., Heymes, C., Belmin, J., Leseche, G., Tedgui, A. (2000).Relation between endothelial cell apoptosis and blood flow direction in human atherosclerotic plaques.Circulation *101*, 2450–3.

Tsuchikura, S., Shoji, T., Shimomura, N., Kakiya, R., Emoto, M., Koyama, H., Ishimura, E., Inaba, M., Nishizawa, Y. (2010). Serum C-reactive protein and thioredoxin levels in subjects with mildly reduced glomerular filtration rate. BMC Nephrol *27*, 11-7.

Tumur, Z., Shimizu, H., Enomoto, A., Miyazaki, H., Niwa, T. (2010). Indoxyl sulfate upregulates expression of ICAM-1 and MCP-1 by oxidative stress-induced NF-kappa B activation. Am. J. Nephrol *31*, 435-41.

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. J. Physiol *552*, 335-344.

Tziomalos, K., Athyros, V. G., Karagiannis, A., Mikhailidis, D. P. (2010). EC dysfunction in metabolic syndrome: prevalence, pathogenesis and management. Nutr Metab Cardiovasc Dis *20*, 140-146.

Ullrich, V., and Bachschmid, M. (2000). Superoxide as a Massenger of Endothelial Function. Biochemical and Biophysical Research Communications *278*, 1-8.

Usatyuk, P. V., Vepa, S., Watkins, T., He, D., Parinandi, N. L., and Natarajan, V. (2003). Redox regulation of reactive oxygen species-induced p38 MAP kinase activation and barrier dysfunction in lung microvascular endothelial cells. Antioxid Redox Signal *5*, 723-730.

Usberti, M., Gerardi, G. M., Bufano, G. (2002). Effects of erythropoietin (EPO) and vitamin E-modified membrane (CL-E) on plasma oxidative stress markers and anemia of hemodialyzed patients. Am J Kidney Dis *40*, 590-9.

Ushio-Fukai, M., Alexander, R, W., Akers, M., Griendling, K. K. (1998). P38 Mitogen-activated protein kinase is a critical component of the redox-sensitive signalling pathways activated by angiotensin II. Role in vascular smooth muscle cell hypertrophy. J Bio Chem *273*, 15022-15029.

Uttara, B., Singh, A. V., Zamboni, P., Mahajan, R. (2009). Oxidative Stress and Neurodegenerative Diseases: A Review of Upstream and Downstream Antioxidant Therapeutic Options. Current Neuropharmacology *7*, 65–74.

Valenti, L., Valenti, G., Como, G., Burdick, L., Santorelli, G., Dongiovanni, P., Rametta, R., Bamonti, F., Novembrino, C., Fracanzani, A. L., Messa, P. G., Fargion S. (2007). HFE gene

mutations and oxidative stress influence serum ferritin, associated with vascular damage, in hemodialysis patients. Am J Nephrol *27*, 101-107.

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M., Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem. Biol. Interact *160*, 1–40.

Van Buul, J. D., van Rijssel, J., van Alphen, F. P., van Stalborch, A. M., Mul, E. P., Hordijk, P. L. (2010). ICAM-1 clustering on endothelial cells recruits VCAM-1. J Biomed Biotechnol 120328.

Van Campenhout, A., Van Campenhout, C., Lagrou, A., Manuel-y-Keenoy, B. (2008). Iron-induced oxidative stress in haemodialysis patients: a pilot study on the impact of diabetes. Biometals *21*, 159-170.

Van Vré, E. A., Ait-Oufella, H., Tedgui, A., Mallat, Z. (2012). Apoptotic cell death and efferocytosis in atherosclerosis.ArteriosclerThrombVascBiol *32*, 887-93.

Van Wyck, D. B., Roppolo, M., Martinez, C. O., Mazey, R. M., McMurray, S. (2005). A randomized, controlled trial comparing IV iron sucrose to oral iron in anemic patients with nondialysis-dependent CKD. Kidney Int *68*, 2846-56.

Van Wyck, D., Anderson, J., Johnson, K. (2004). Labile iron in parenteral iron formulations: a quantitative and comparative study. Nephrol Dial Transplant *19*, 561-5.

VanderLaan, P. A., Reardon, C. A., Getz, G. S. (2004). Site specificity of atherosclerosis: site-selective responses to atherosclerotic modulators. Arterioscler Thromb Vasc Biol *24*,12-22.

Varani, J., Dame, M. K., Taylor, C. G, Sarma, V., Merino, R., Kunkel, R. G., Nunez, G., Dixit, V. M. (1995). Age-dependent injury in human umbilical vein endothelial cells: relationship to apoptosis and correlation with a lack of A20 expression.Lab Invest *73*, 851-8.

Vaziri, N. D. (2004). Oxidative stress in uremia: nature, mechanisms, and potential consequences. Semin Nephrol *24*, 469-473.

Vaziri, N. D. (2012). Understanding iron: promoting its safe use in patients with chronic kidney failure treated by hemodialysis. Am J Kidney Dis *10*, 027.

Viaro, F., Nobre, F., and Evora, P. R. B. (2000). “Expression of nitric oxide synthases in the pathophysiology of cardiovascular diseases,” Arquivos Brasileiros de Cardiologia *74*, 380-393.

Vita, J. A., and Keaney, J. F. (2002). Endothelial function: a barometer for cardiovascular risk? Circulation *106*, 640-642.

Vlagopoulos, P. T., Tighiouart, H., Weiner, D. E., Griffith, J., Pettitt, D., Salem, D. N., Levey, A. S., Sarnak, M. J. (2005). Anemia as a risk factor for cardiovascular disease and all-cause mortality in diabetes: the impact of chronic kidney disease. J Am Soc Nephrol *16*, 3403-3410.

Voelkel, N. F., and Rounds, S. (2009). The Pulmonary Endothelium: Function in health and disease. New York: Wiley & Sons.

Vogiatzil, G., Tousoulis, D., Stefanadis, C. (2009). The role of oxidative stress in atherosclerosis. Hellenic J Cardiol *50*, 402-9.

Wada, T., and Penninger, J. M. (2004). Mitogen-activated protein kinases in apoptosis regulation. Oncogene *23*, 2838-2849.

Wagener, F. A., Carels, C. E., Lundvig, D. M..(2013). Targeting the Redox Balance in Inflammatory Skin Conditions.Int J Mol Sci *14*, 9126-67.

Wagner, E. F., and Nebreda, A. R. (2009). Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer *9*, 537-549.

Wang, K., Yin, X. M., Chao, D. T., Milliman, C. L., Korsmeyer, S. J. (1996). BID: a novel BH3 domain-only death agonist. Genes Dev *10*, 2859-2869.

Wang, S., Geraci, G., Kuhlmann, M. K., Levin, N. W., Handelman, G. J. (2008). Chemical reactions of vitamin C with intravenous-iron formulations. Nephrol Dial Transplant *23*, 120-5.

Wang, T., Chiang, E.T., Moreno-Vinasco, L., Lang, G. D., Pendyala, S., Samet, J. M., Geyh, A. S., Breysse, P. N., Chillrud, S. N., Natarajan, V., Garcia, J. G. (2010). Particulate matter disrupts human lung endothelial barrier integrity via ROS- and p38 MAPK-dependent pathways. Am J Respir Cell Mol Biol *42*, 442-9.

Wang, X. (2001). The expanding role of mitochondria in apoptosis. Genes Dev *15*, 922–2933.

Wang, Y., Huang, S., Sah, V. P., Ross, J., Jr., Brown, J. H., Han, J., Chien, K. R. (1998). Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogenactivated protein kinase family. J BiolChem *273*, 2161-2168.

Warboys, C. M., Amini, N., de Luca, A., Evans, P. C. (2011). The role of blood flow in determining the sites of atherosclerotic plaques. F1000 Med Rep *3*, 5.

Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., Korsmeyer, S. J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science *292*, 727-730.

Weigand, M. A., Plachky, J., Thies, J. C., Spies-Martin, D., Otto, G., Martin, E., Bardenheuer, H. J. (2001). N-acetyl-cysteine attenuates the increase in alpha-glutathione S-transferase and circulating ICAM-1 and VCAM-1 after reperfusion in humans undergoing liver transplantation. Transplantation *72*, 694-8.

Weinberg, E. D. (1990). Cellular iron metabolism in health and disease. Drug Metab. Rev *22*, 531-579.

Weiner, D. E., Tighiouart, H., Stark, P. C., Amin, M. G., MacLeod, B., Griffith, J. L., Salem, D. N., Levey, A. S., Sarnak, M. J. (2004). Kidney disease as a risk factor for recurrent cardiovascular disease and mortality. Am J Kidney Dis *44*, 198-206.

Weiss, N., Heydrick, S., Zhang, Y. Y., Bierl, C., Cap, A., Loscalzo, J. (2002). Cellular redox state and EC dysfunction in mildly hyperhomocysteine miccystathionine beta-synthase-deficient mice.ArteriosclerThrombVascBiol *22*, 34-41.

Wen, J., You, K. R., Lee, S. Y., Song, C. H., Kim, D. G. (2002). Oxidative stress-mediated apoptosis.The anticancer effect of the sesquiterpene lactone parthenolide.J BiolChem *277*, 38954-64.

Wink, D. A., and Mitchell, J. B. (1998). Chemical biology of nitric oxide: insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. Free Rad Biol Med *25*, 434-456.

Witko-Sarsat, V., Friedlander, M., Nguyen Khoa, T. (1998). Advanced oxidation protein products as novel mediators of inflammation and monocyte activation in chronic renal failure. J Immunol *161*, 2524-32.

Woodrow, P. (2007a). Caring for patients receiving oxygen therapy. Nurs Older People *19*, 31-5.

World Health Organization. (2005). Preventing chronic diseases: a vital investment: WHO global report. Geneva; Ref Type: Report.

Wu-Wong, J. R. (2008). Endothelial dysfunction and chronic kidney disease: treatment options. Curr Opin Investig Drugs *9,* 970-82.

Xu, D., Neville, R., Finkel, T. (2000). Homocysteine accelerates endothelial cell senescence. FEBS Lett *470*, 20-24.

Xu, H., Zaidi, M., Struve, J., Jones, D. W., Krolikowski, J. G. (2011). Abnormal fibrillin-1 expression and chronic oxidative stress mediate endothelial mesenchymal transition in a murine model of systemic sclerosis. Am J Physiol Cell Physiol *300*, 550-6.

Xu, N., Rahman, A., Minshall, R. D., Tiruppathi, C., and Malik, A. (2000). Beta(2)-integrin blockade driven by E-selectin promoter prevents neutrophil sequestration and lung injury in mice. Circ Res *87*, 254-260.

Yang, B., Oo, T. N., Rizzo, V. (2006). Lipid rafts mediate H2O2 prosurvival effects in cultured endothelial cells. FASEB J *20*, 1501-3.

Yang, J. K., Wang, L., Zheng, L., Wan, F., Ahmed, M., Lenardo, M. J., Wu, H. (2005). Crystal structure of MC159 reveals molecular mechanism of DISC assembly and FLIP inhibition. Mol Cell *20*, 939-949.

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., Wang, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science *275*, 1129-1132.

Yang, Y., Liu, X., Huang, J., Zhong, Y., Mao, Z., Xiao, H., Zhuo, Y. (2012). Inhibition of p38 mitogen-activated protein kinase phosphorylation decrease tert-butyl hydroperoxide-induced apoptosis in human trabecular meshwork cells. Molecular Vision *18*, 2127-2136.

Ye, J., Li, J., Yu, Y., Wei, Q., Deng, W., Yu, L. (2010). L-carnitine attenuates oxidant injury in HK-2 cells via ROS-mitochondria pathway. Regul Pept *161*, 58-66.

Yessayan, L., Yee, J., Zasuwa, G., Frinak, S., Besarab, A. (2014). Iron repletion is associated with reduction in platelet counts in non-dialysis chronic kidney disease patients independent of erythropoiesis-stimulating agent use: a retrospective cohort study. BMC Nephrol *19*, 15:119.

Youle, R. J and Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol *9*, 47–59.

Yu, W., Akishita, M., Xi, H., Nagai, K., Sudoh, N., Hasegawa, H., Kozaki, K., Toba, K. (2006). Angiotensin converting enzyme inhibitor attenuates oxidative stress-induced endothelial cell apoptosis via p38 MAP kinase inhibition. Clin Chim Acta *364*, 328-34.

Yu, X., Acehan, D., Menetret, J. F., Booth, C. R. (2005). A structure of the human apoptosome at 12.8 A resolution provides insights into this cell death platform. Structure *13*, 1725–1735.

Yuan, J., Lipinski, M., Degterev, A. (2003).Diversity in the mechanisms of neuronal cell death. Neuron *40*, 401–413.

Yue, T. L., Ni, J., Romanic, A. M., Gu, J. L., Keller, P., Wang, C., Kumar, S., Yu, G. L., Hart, T. K., Wang, X., Xia, Z., DeWolf, W. E. Jr., Feuerstein, G. Z. (1999). TL1, a novel tumor necrosis factor-like cytokine, induces apoptosis in endothelial cells. Involvement of activation of stress protein kinases (stress-activated protein kinase and p38 mitogen-activated protein kinase) and caspase-3-like protease. J Biol Chem *274*, 1479-1486.

Yuksel, A., Kebapcilar, L., Erdur, E., Bozkaya, G., Sari, I., Alacacioglu, A., Kebapcilar, A. G., Sop, G. (2010). The effect of iron treatment on adhesion molecules in patients with iron deficiency anemia. Biol Trace Elem Res. 2010 Dec *137*, 317-23.

Zager, R. A., Johnson, A. C., Hanson, S. Y. (2004). Parental iron nephrotoxicity: Potential mechanisms and consequences. Kidney Int *66*, 144–156.

Zager, R. A., Johnson, A. C., Hanson, S. Y., Wasse, H. (2002). Parenteral iron formulations: A comparative toxicologic analysis and mechanisms of cell injury. Am J Kidney Dis *40*, 90-103.

Zalba, G., San Jose, G., Moreno, M. U., Fortuno, M. A., Beaumont, F. J., Diez, J. (2001). Oxidative stress in arterial hypertension: role of NAD(P)H oxidase. Hypertension *38*, 1395-1399.

Zechner, D., Craig, R., Hanford, D. S., McDonough, P. M., Sabbadini, R. A., Glembotski, C. C. (1998). MKK6 activates myocardial cell NF-kappa B and inhibits apoptosis in a p38 mitogen-activated protein kinase-dependent manner. J. Biol. Chem *273*, 8232-8239.

Zha, H., and Reed, J. C. (1997). Heterodimerization-independent functions of cell death regulatory proteins Bax and Bcl-2 in yeast and mammalian cells. J Biol Chem *272*, 31482-8.

Zhang, C., Cai, Y., Adachi, M. T., Oshiro, S., Aso, T., Kaufman, R. J., and Kitajima, S. (2001a). Homocysteine induces programmed cell death in human vascular endothelial cells through activation of the unfolded protein response. J BiolChem *276*, 35867-35874.

Zhang, D. L., Liu, J., Liu, S., Zhang, Y., Liu, W. H. (2010). The differences of asymmetric dimethylarginine removal by different dialysis treatments. Ren. Fail *32*, 935-940.

Zhang, D. X., and Gutterman, D. D. (2007). Mitochondrial reactive oxygen species-mediated signaling in endothelial cells. Am J Physiol Heart Circ Physiol *292*, 2023-2031.

Zhang, J., Defelice, A. F., Hanig, J. P., Colatsky, T. (2010). Biomarkers of endothelial cell activation serve as potential surrogate markers for drug-induced vascular injury. Toxicol Pathol *38*, 856-71.

Zhang, K. Y., and Zuo, L. (2014). Vitamin C supplementation in patients on maintenance dialysis. World J Clin Urol *3*, 344-350.

Zhang, K., Li, Y., Cheng, X., Liu, L., Bai, W., Guo, W., Wu, L., Zuo, L. (2013). Cross-over study of influence of oral vitamin C supplementation on inflammatory status in maintenance hemodialysispatients. BMC Nephrol *14*, 252.

Zhang, X., Zhao, X. D., Shi, J. X., Yin, H. X. (2011). Inhibition of the p38 mitogen-activated protein kinase (MAPK) pathway attenuates cerebral vasospasm following experimental subarachnoid hemorrhage in rabbits. Ann Clin Lab Sci *41*, 244-50.

Zheng, H., Huang, X., Zhang, Q., Katz, S. D. (2006). Iron sucrose augments homocysteine-induced EC dysfunction in normal subjects. Kidney Int *69,* 679-684.

Zimmermann, K. C., and .Green, D. R. (2001). "How cells die: apoptosis pathways." J Allergy ClinImmunol *108*, 99-103.

Zoccali, C., Bode-Böger, S., Mallamaci, F. (2001). Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study. Lancet *358*, 2113-7.

Zoccali, C., Mallamaci, F., Tripepi, G. (2002). Adiponectin, metabolic risk factors and cardiovascular events among patients with end-stage renal disease. J Am Soc Nephrol *13*, 134-141.

APPENDIX 1

Presentation of data and conference

Hadeiba, T. H. A., Thornton, J., Graham, A. M. Endothelial cell injury and adhesion molecule expression mediated by intravenous iron preparations *in vitro*. Faculty of Life Sciences, Research Open Day, University of Bradford, UK. (Poster), 2nd April 2014.

Hadeiba, T. H. A., Thornton, J., Graham, A. M. Endothelial cell injury and adhesion molecule expression mediated by intravenous iron preparations *in vitro*. Leeds Institute of Cardiovascular and Metabolic Medicine (LICAMM), Early Career Research Conference in Cardiovascular and Diabetes Research, University of Leeds, UK. (Poster), 10th-11th September 2014.

Hadeiba, T. H. A., and Graham, A. M. Intravenous iron preparations promote endothelial cell damage and adhesion molecule expression *in vitro*. Faculty of Life Sciences, Research and Development Open Day, University of Bradford, UK. (Poster), 28th May 2015.

APPENDIX 2

Preliminary experiments to demonstrate that exposure ROS causes phosphorylation of p38 MAPK in EA.hy926 endothelial cell line.

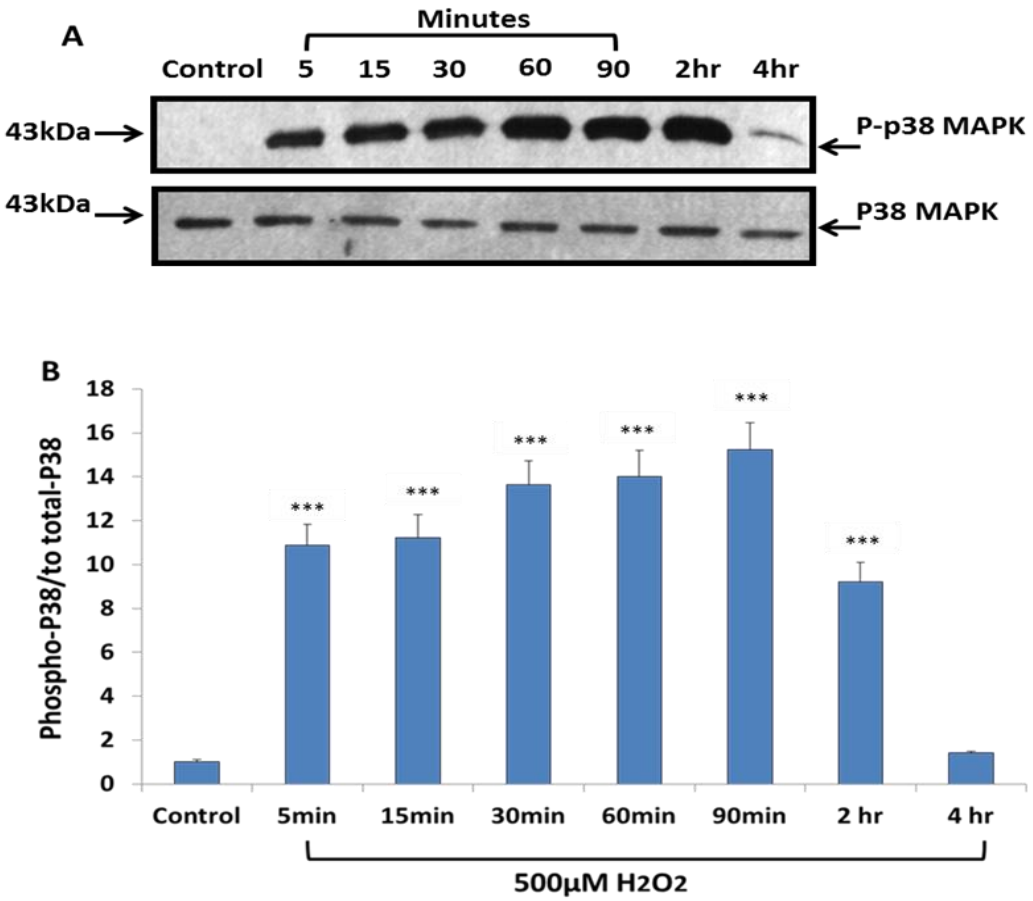


Figure: Shows the effects of H_2O_2 on p38 MAPK phosphorylation in EA.hy926 endothelial cell line. EA.hy926 were cultured in 6 well plates until confluence, then cells were treated with 500 µM of H_2O_2 for different time course 5, 15, 30, 60, 90 min, 2hr and 4hr, and lysed. 30 µg proteins from each sample was loaded on an SDS-PAGE gel and used in the Western blot. **(A):** representative blots; the phospho-p38 and total p38 MAPK proteins were detected using specific antibodies and were identified by Western blot as a band of approximately 43 kDa.**(B):** the phospho-p38/total p38 ratio were quantified by densitometry and expressed as the ratio compared to the control. All values are expressed as mean (+/-SD) from three different experiments (n=3). ****P*<0.001 versus control.